receptor by competing with it for free ligand (e.g., soluble TNF receptors used in reducing inflammation), or to bring about a desired response (e.g., blood vessel growth).

Similarly, antibodies directed to follistatin-3 polypeptides can also be used to treat disease. For example, administration of an antibody directed to a follistatin-3 polypeptide can bind and reduce overproduction of the polypeptide. Similarly, administration of an antibody can activate the polypeptide, such as by binding to a polypeptide bound to a membrane (receptor).

At the very least, the follistatin-3 polypeptides can be used as molecular weight markers on SDS-PAGE gels or on molecular sieve gel filtration columns using methods well known to those of skill in the art. Follistatin-3 polypeptides can also be used to raise antibodies, which in turn are used to measure protein expression from a recombinant cell, as a way of assessing transformation of the host cell. Moreover, follistatin-3 polypeptides can be used to test the following biological activities.

Gene Therapy Methods.

Another aspect of the present invention is to gene therapy methods for treating disorders, diseases and conditions. The gene therapy methods relate to the introduction of nucleic acid (DNA, RNA and antisense DNA or RNA) sequences into an animal to achieve expression of the follistatin-3 polypeptide of the present invention. This method requires a polynucleotide which codes for a follistatin-3 polypeptide operatively linked to a promoter and any other genetic elements necessary for the expression of the polypeptide by the target tissue. Such gene therapy and delivery techniques are known in the art, see, for example, WO90/11092, which is herein incorporated by reference.

Thus, for example, cells from a patient may be engineered with a polynucleotide (DNA or RNA) comprising a promoter operably linked to a follistatin-3 polynucleotide ex vivo, with the engineered cells then being provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, see Belldegrun, A., et al., J. Natl. Cancer Inst. 85: 207-216 (1993); Ferrantini, M. et al., Cancer Research 53: 1107-1112 (1993); Ferrantini, M. et al., J. Immunology 153: 4604-4615 (1994); Kaido, T., et al., Int. J. Cancer 60: 221-229 (1995); Ogura, H., et al., Cancer Research

50: 5102-5106 (1990); Santodonato, L., et al., Human Gene Therapy 7:1-10 (1996); Santodonato, L., et al., Gene Therapy 4:1246-1255 (1997); and Zhang, J.-F. et al., Cancer Gene Therapy 3: 31-38 (1996)), which are herein incorporated by reference. In one embodiment, the cells which are engineered are arterial cells. The arterial cells may be reintroduced into the patient through direct injection to the artery, the tissues surrounding the artery, or through catheter injection.

As discussed in more detail below, the follistatin-3 polynucleotide constructs can be delivered by any method that delivers injectable materials to the cells of an animal, such as, injection into the interstitial space of tissues (heart, muscle, skin, lung, liver, and the like). The follistatin-3 polynucleotide constructs may be delivered in a pharmaceutically acceptable liquid or aqueous carrier.

In one embodiment, the follistatin-3 polynucleotide is delivered as a naked polynucleotide. The term "naked" polynucleotide, DNA or RNA refers to sequences that are free from any delivery vehicle that acts to assist, promote or facilitate entry into the cell, including viral sequences, viral particles, liposome formulations, lipofectin or precipitating agents and the like. However, the follistatin-3 polynucleotides can also be delivered in liposome formulations and lipofectin formulations and the like can be prepared by methods well known to those skilled in the art. Such methods are described, for example, in U.S. Patent Nos. 5,593,972, 5,589,466, and 5,580,859, which are herein incorporated by reference.

The follistatin-3 polynucleotide vector constructs used in the gene therapy method are preferably constructs that will not integrate into the host genome nor will they contain sequences that allow for replication. Appropriate vectors include pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; pSVK3, pBPV, pMSG and pSVL available from Pharmacia; and pEF1/V5, pcDNA3.1, and pRc/CMV2 available from Invitrogen. Other suitable vectors will be readily apparent to the skilled artisan.

Any strong promoter known to those skilled in the art can be used for driving the expression of follistatin-3 DNA. Suitable promoters include adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter, the respiratory syncytial virus (RSV) promoter,

inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoAI promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs; the b-actin promoter; and human growth hormone promoters. The promoter also may be the native promoter for follistatin-3.

Unlike other gene therapy techniques, one major advantage of introducing naked nucleic acid sequences into target cells is the transitory nature of the polynucleotide synthesis in the cells. Studies have shown that non-replicating DNA sequences can be introduced into cells to provide production of the desired polypeptide for periods of up to six months.

The follistatin-3 polynucleotide construct can be delivered to the interstitial space of tissues within the an animal, including of muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, and connective tissue. Interstitial space of the tissues comprises the intercellular, fluid, mucopolysaccharide matrix among the reticular fibers of organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation and the lymph fluid of the lymphatic channels. Delivery to the interstitial space of muscle tissue is preferred for the reasons discussed below. They may be conveniently delivered by injection into the tissues comprising these cells. They are preferably delivered to and expressed in persistent, non-dividing cells which are differentiated, although delivery and expression may be achieved in non-differentiated or less completely differentiated cells, such as, for example, stem cells of blood or skin fibroblasts. In vivo muscle cells are particularly competent in their ability to take up and express polynucleotides.

For the naked acid sequence injection, an effective dosage amount of DNA or RNA will be in the range of from about 0.05 mg/kg body weight to about 50 mg/kg body weight. Preferably the dosage will be from about 0.005 mg/kg to about 20 mg/kg and more preferably from about 0.05 mg/kg to about 5 mg/kg. Of course, as the artisan of ordinary skill will appreciate, this dosage will vary according to the tissue site of

injection. The appropriate and effective dosage of nucleic acid sequence can readily be determined by those of ordinary skill in the art and may depend on the condition being treated and the route of administration.

The preferred route of administration is by the parenteral route of injection into the interstitial space of tissues. However, other parenteral routes may also be used, such as, inhalation of an aerosol formulation particularly for delivery to lungs or bronchial tissues, throat or mucous membranes of the nose. In addition, naked follistatin-3 DNA constructs can be delivered to arteries during angioplasty by the catheter used in the procedure.

The naked polynucleotides are delivered by any method known in the art, including, but not limited to, direct needle injection at the delivery site, intravenous injection, topical administration, catheter infusion, and so-called "gene guns". These delivery methods are known in the art.

As is evidenced in the Examples, naked follistatin-3 nucleic acid sequences can be administered in vivo results in the successful expression of follistatin-3 polypeptide in the femoral arteries of rabbits.

The constructs may also be delivered with delivery vehicles such as viral sequences, viral particles, liposome formulations, lipofectin, precipitating agents, etc. Such methods of delivery are known in the art.

In certain embodiments, the follistatin-3 polynucleotide constructs are complexed in a liposome preparation. Liposomal preparations for use in the instant invention include cationic (positively charged), anionic (negatively charged) and neutral preparations. However, cationic liposomes are particularly preferred because a tight charge complex can be formed between the cationic liposome and the polyanionic nucleic acid. Cationic liposomes have been shown to mediate intracellular delivery of plasmid DNA (Felgner et al., Proc. Natl. Acad. Sci. USA (1987) 84:7413-7416, which is herein incorporated by reference); mRNA (Malone et al., Proc. Natl. Acad. Sci. USA (1989) 86:6077-6081, which is herein incorporated by reference); and purified transcription factors (Debs et al., J. Biol. Chem. (1990) 265:10189-10192, which is herein incorporated by reference), in functional form.

Cationic liposomes are readily available. For example, N[1-2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA) liposomes are particularly useful and are available under the trademark Lipofectin, from GIBCO BRL, Grand Island, N.Y. (See, also, Felgner et al., Proc. Natl Acad. Sci. USA (1987) 84:7413-7416, which is herein incorporated by reference). Other commercially available liposomes include transfectace (DDAB/DOPE) and DOTAP/DOPE (Boehringer).

Other cationic liposomes can be prepared from readily available materials using techniques well known in the art. See, e.g. PCT Publication No. WO 90/11092 (which is herein incorporated by reference) for a description of the synthesis of DOTAP (1,2-bis(oleoyloxy)-3-(trimethylammonio)propane) liposomes. Preparation of DOTMA liposomes is explained in the literature, see, e.g., P. Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413-7417, which is herein incorporated by reference. Similar methods can be used to prepare liposomes from other cationic lipid materials.

Similarly, anionic and neutral liposomes are readily available, such as from Avanti Polar Lipids (Birmingham, Ala.), or can be easily prepared using readily available materials. Such materials include phosphatidyl, choline, cholesterol, phosphatidyl ethanolamine, dioleoylphosphatidyl choline (DOPC), dioleoylphosphatidyl glycerol (DOPG), dioleoylphoshatidyl ethanolamine (DOPE), among others. These materials can also be mixed with the DOTMA and DOTAP starting materials in appropriate ratios. Methods for making liposomes using these materials are well known in the art.

For example, commercially dioleoylphosphatidyl choline (DOPC), dioleoylphosphatidyl glycerol (DOPG), and dioleoylphosphatidyl ethanolamine (DOPE) can be used in various combinations to make conventional liposomes, with or without the addition of cholesterol. Thus, for example, DOPG/DOPC vesicles can be prepared by drying 50 mg each of DOPG and DOPC under a stream of nitrogen gas into a sonication vial. The sample is placed under a vacuum pump overnight and is hydrated the following day with deionized water. The sample is then sonicated for 2 hours in a capped vial, using a Heat Systems model 350 sonicator equipped with an inverted cup (bath type) probe at the maximum setting while the bath is circulated at 15EC. Alternatively, negatively charged vesicles can be prepared without sonication to produce multilamellar

vesicles or by extrusion through nucleopore membranes to produce unilamellar vesicles of discrete size. Other methods are known and available to those of skill in the art.

The liposomes can comprise multilamellar vesicles (MLVs), small unilamellar vesicles (SUVs), or large unilamellar vesicles (LUVs), with SUVs being preferred. The various liposome-nucleic acid complexes are prepared using methods well known in the art. See, e.g., Straubinger et al., Methods of Immunology (1983), 101:512-527, which is herein incorporated by reference. For example, MLVs containing nucleic acid can be prepared by depositing a thin film of phospholipid on the walls of a glass tube and subsequently hydrating with a solution of the material to be encapsulated. SUVs are prepared by extended sonication of MLVs to produce a homogeneous population of unilamellar liposomes. The material to be entrapped is added to a suspension of preformed MLVs and then sonicated. When using liposomes containing cationic lipids, the dried lipid film is resuspended in an appropriate solution such as sterile water or an isotonic buffer solution such as 10 mM Tris/NaCl, sonicated, and then the preformed liposomes are mixed directly with the DNA. The liposome and DNA form a very stable complex due to binding of the positively charged liposomes to the cationic DNA. SUVs find use with small nucleic acid fragments. LUVs are prepared by a number of methods, well known in the art. Commonly used methods include Ca^{2+}-EDTA chelation (Papahadjopoulos et al., Biochim. Biophys. Acta (1975) 394:483; Wilson et al., Cell (1979) 17:77); ether injection (Deamer, D. and Bangham, A., Biochim. Biophys. Acta (1976) 443:629; Ostro et al., Biochem. Biophys. Res. Commun. (1977) 76:836; Fraley et al., Proc. Natl. Acad. Sci. USA (1979) 76:3348); detergent dialysis (Enoch, H. and Strittmatter, P., Proc. Natl. Acad. Sci. USA (1979) 76:145); and reverse-phase evaporation (REV) (Fraley et al., J. Biol. Chem. (1980) 255:10431; Szoka, F. and Papahadjopoulos, D., Proc. Natl. Acad. Sci. USA (1978) 75:145; Schaefer-Ridder et al., Science (1982) 215:166), which are herein incorporated by reference.

Generally, the ratio of DNA to liposomes will be from about 10:1 to about 1:10. Preferably, the ration will be from about 5:1 to about 1:5. More preferably, the ration will be about 3:1 to about 1:3. Still more preferably, the ratio will be about 1:1.

U.S. Patent No. 5,676,954 (which is herein incorporated by reference) reports on the injection of genetic material, complexed with cationic liposomes carriers, into mice.

U.S. Patent Nos. 4,897,355, 4,946,787, 5,049,386, 5,459,127, 5,589,466, 5,693,622, 5,580,859, 5,703,055, and international publication no. WO 94/9469 (which are herein incorporated by reference) provide cationic lipids for use in transfecting DNA into cells and mammals. U.S. Patent Nos. 5,589,466, 5,693,622, 5,580,859, 5,703,055, and international publication no. WO 94/9469 (which are herein incorporated by reference) provide methods for delivering DNA-cationic lipid complexes to mammals.

In certain embodiments, cells are be engineered, ex vivo or in vivo, using a retroviral particle containing RNA which comprises a sequence encoding follistatin-3. Retroviruses from which the retroviral plasmid vectors may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, Rous sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, Myeloproliferative Sarcoma Virus, and mammary tumor virus.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, R-2, R-AM, PA12, T19-14X, VT-19-17-H2, RCRE, RCRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, Human Gene Therapy 1:5-14 (1990), which is incorporated herein by reference in its entirety. The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and $CaPO_4$ precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

The producer cell line generates infectious retroviral vector particles which include polynucleotide encoding follistatin-3. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either in vitro or in vivo. The transduced eukaryotic cells will express follistatin-3.

In certain other embodiments, cells are engineered, ex vivo or in vivo, with follistatin-3 polynucleotide contained in an adenovirus vector. Adenovirus can be manipulated such that it encodes and expresses follistatin-3, and at the same time is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. Adenovirus expression is achieved without integration of the viral DNA into the host cell chromosome, thereby alleviating concerns about insertional mutagenesis. Furthermore,

adenoviruses have been used as live enteric vaccines for many years with an excellent safety profile (Schwartz, A. R. et al. (1974) Am. Rev. Respir. Dis.109:233-238). Finally, adenovirus mediated gene transfer has been demonstrated in a number of instances including transfer of alpha-1-antitrypsin and CFTR to the lungs of cotton rats (Rosenfeld, M. A. et al. (1991) Science 252:431-434; Rosenfeld et al., (1992) Cell 68:143-155). Furthermore, extensive studies to attempt to establish adenovirus as a causative agent in human cancer were uniformly negative (Green, M. et al. (1979) Proc. Natl. Acad. Sci. USA 76:6606).

Suitable adenoviral vectors useful in the present invention are described, for example, in Kozarsky and Wilson, Curr. Opin. Genet. Devel. 3:499-503 (1993); Rosenfeld et al., Cell 68:143-155 (1992); Engelhardt et al., Human Genet. Ther. 4:759-769 (1993); Yang et al., Nature Genet. 7:362-369 (1994); Wilson et al., Nature 365:691-692 (1993); and U.S. Patent No. 5,652,224, which are herein incorporated by reference. For example, the adenovirus vector Ad2 is useful and can be grown in human 293 cells. These cells contain the E1 region of adenovirus and constitutively express Ela and Elb, which complement the defective adenoviruses by providing the products of the genes deleted from the vector. In addition to Ad2, other varieties of adenovirus (e.g., Ad3, Ad5, and Ad7) are also useful in the present invention.

Preferably, the adenoviruses used in the present invention are replication deficient. Replication deficient adenoviruses require the aid of a helper virus and/or packaging cell line to form infectious particles. The resulting virus is capable of infecting cells and can express a polynucleotide of interest which is operably linked to a promoter, for example, the HARP promoter of the present invention, but cannot replicate in most cells. Replication deficient adenoviruses may be deleted in one or more of all or a portion of the following genes: E1a, E1b, E3, E4, E2a, or L1 through L5.

In certain other embodiments, the cells are engineered, ex vivo or in vivo, using an adeno-associated virus (AAV). AAVs are naturally occurring defective viruses that require helper viruses to produce infectious particles (Muzyczka, N., Curr. Topics in Microbiol. Immunol. 158:97 (1992)). It is also one of the few viruses that may integrate its DNA into non-dividing cells. Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate, but space for exogenous DNA is limited to about 4.5

kb. Methods for producing and using such AAVs are known in the art. See, for example, U.S. Patent Nos. 5,139,941, 5,173,414, 5,354,678, 5,436,146, 5,474,935, 5,478,745, and 5,589,377.

For example, an appropriate AAV vector for use in the present invention will include all the sequences necessary for DNA replication, encapsidation, and host-cell integration. The follistatin-3 polynucleotide construct is inserted into the AAV vector using standard cloning methods, such as those found in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press (1989). The recombinant AAV vector is then transfected into packaging cells which are infected with a helper virus, using any standard technique, including lipofection, electroporation, calcium phosphate precipitation, etc. Appropriate helper viruses include adenoviruses, cytomegaloviruses, vaccinia viruses, or herpes viruses. Once the packaging cells are transfected and infected, they will produce infectious AAV viral particles which contain the follistatin-3 polynucleotide construct. These viral particles are then used to transduce eukaryotic cells, either ex vivo or in vivo. The transduced cells will contain the follistatin-3 polynucleotide construct integrated into its genome, and will express follistatin-3.

Another method of gene therapy involves operably associating heterologous control regions and endogenous polynucleotide sequences (e.g. encoding follistatin-3) via homologous recombination (see, e.g., U.S. Patent No. 5,641,670, issued June 24, 1997; International Publication No. WO 96/29411, published September 26, 1996; International Publication No. WO 94/12650, published August 4, 1994; Koller et al., Proc. Natl. Acad. Sci. USA 86:8932-8935 (1989); and Zijlstra et al., Nature 342:435-438 (1989). This method involves the activation of a gene which is present in the target cells, but which is not normally expressed in the cells, or is expressed at a lower level than desired.

Polynucleotide constructs are made, using standard techniques known in the art, which contain the promoter with targeting sequences flanking the promoter. Suitable promoters are described herein. The targeting sequence is sufficiently complementary to an endogenous sequence to permit homologous recombination of the promoter-targeting sequence with the endogenous sequence. The targeting sequence will be sufficiently

near the 5' end of the follistatin-3 desired endogenous polynucleotide sequence so the promoter will be operably linked to the endogenous sequence upon homologous recombination.

The promoter and the targeting sequences can be amplified using PCR. Preferably, the amplified promoter contains distinct restriction enzyme sites on the 5' and 3' ends. Preferably, the 3' end of the first targeting sequence contains the same restriction enzyme site as the 5' end of the amplified promoter and the 5' end of the second targeting sequence contains the same restriction site as the 3' end of the amplified promoter. The amplified promoter and targeting sequences are digested and ligated together.

The promoter-targeting sequence construct is delivered to the cells, either as naked polynucleotide, or in conjunction with transfection-facilitating agents, such as liposomes, viral sequences, viral particles, whole viruses, lipofection, precipitating agents, etc., described in more detail above. The P promoter-targeting sequence can be delivered by any method, included direct needle injection, intravenous injection, topical administration, catheter infusion, particle accelerators, etc. The methods are described in more detail below.

The promoter-targeting sequence construct is taken up by cells. Homologous recombination between the construct and the endogenous sequence takes place, such that an endogenous follistatin-3 sequence is placed under the control of the promoter. The promoter then drives the expression of the endogenous follistatin-3 sequence.

The polynucleotides encoding follistatin-3 may be administered along with other polynucleotides encoding other angiongenic proteins. Angiogenic proteins include, but are not limited to, acidic and basic fibroblast growth factors, VEGF-1, epidermal growth factor alpha and beta, platelet-derived endothelial cell growth factor, platelet-derived growth factor, tumor necrosis factor alpha, hepatocyte growth factor, insulin like growth factor, colony stimulating factor, macrophage colony stimulating factor, granulocyte/macrophage colony stimulating factor, and nitric oxide synthase.

Preferably, the polynucleotide encoding follistatin-3 contains a secretory signal sequence that facilitates secretion of the protein. Typically, the signal sequence is positioned in the coding region of the polynucleotide to be expressed towards or at the 5'

end of the coding region. The signal sequence may be homologous or heterologous to the polynucleotide of interest and may be homologous or heterologous to the cells to be transfected. Additionally, the signal sequence may be chemically synthesized using methods known in the art.

Any mode of administration of any of the above-described polynucleotides constructs can be used so long as the mode results in the expression of one or more molecules in an amount sufficient to provide a therapeutic effect. This includes direct needle injection, systemic injection, catheter infusion, biolistic injectors, particle accelerators (i.e., "gene guns"), gelfoam sponge depots, other commercially available depot materials, osmotic pumps (e.g., Alza minipumps), oral or suppositorial solid (tablet or pill) pharmaceutical formulations, and decanting or topical applications during surgery. For example, direct injection of naked calcium phosphate-precipitated plasmid into rat liver and rat spleen or a protein-coated plasmid into the portal vein has resulted in gene expression of the foreign gene in the rat livers (Kaneda et al., Science 243:375 (1989)).

A preferred method of local administration is by direct injection. Preferably, a recombinant molecule of the present invention complexed with a delivery vehicle is administered by direct injection into or locally within the area of arteries. Administration of a composition locally within the area of arteries refers to injecting the composition centimeters and preferably, millimeters within arteries.

Another method of local administration is to contact a polynucleotide construct of the present invention in or around a surgical wound. For example, a patient can undergo surgery and the polynucleotide construct can be coated on the surface of tissue inside the wound or the construct can be injected into areas of tissue inside the wound.

Therapeutic compositions useful in systemic administration, include recombinant molecules of the present invention complexed to a targeted delivery vehicle of the present invention. Suitable delivery vehicles for use with systemic administration comprise liposomes comprising ligands for targeting the vehicle to a particular site.

Preferred methods of systemic administration, include intravenous injection, aerosol, oral and percutaneous (topical) delivery. Intravenous injections can be performed using methods standard in the art. Aerosol delivery can also be performed

using methods standard in the art (see, for example, Stribling et al., Proc. Natl. Acad. Sci. USA 189:11277-11281, 1992, which is incorporated herein by reference). Oral delivery can be performed by complexing a polynucleotide construct of the present invention to a carrier capable of withstanding degradation by digestive enzymes in the gut of an animal. Examples of such carriers, include plastic capsules or tablets, such as those known in the art. Topical delivery can be performed by mixing a polynucleotide construct of the present invention with a lipophilic reagent (e.g., DMSO) that is capable of passing into the skin.

Determining an effective amount of substance to be delivered can depend upon a number of factors including, for example, the chemical structure and biological activity of the substance, the age and weight of the animal, the precise condition requiring treatment and its severity, and the route of administration. The frequency of treatments depends upon a number of factors, such as the amount of polynucleotide constructs administered per dose, as well as the health and history of the subject. The precise amount, number of doses, and timing of doses will be determined by the attending physician or veterinarian.

Therapeutic compositions of the present invention can be administered to any animal, preferably to mammals and birds. Preferred mammals include humans, dogs, cats, mice, rats, rabbits sheep, cattle, horses and pigs, with humans being particularly preferred.

Biological Activities of Follistatin-3.

Follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, can be used in assays to test for one or more biological activities. If follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, do exhibit activity in a particular assay, it is likely that follistatin-3 may be involved in the diseases associated with the biological activity. Therefore, follistatin-3 could be used to treat the associated disease.

Immune Activity

Follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3 may be useful in treating deficiencies or disorders of the immune system, by activating or inhibiting the proliferation, differentiation, or mobilization (chemotaxis) of immune cells. Immune cells develop through a process called hematopoiesis, producing myeloid (platelets, red blood cells, neutrophils, and macrophages) and lymphoid (B and T lymphocytes) cells from pluripotent stem cells. The etiology of these immune deficiencies or disorders may be genetic, somatic, such as cancer or some autoimmune disorders, acquired (e.g., by chemotherapy or toxins), or infectious. Moreover, follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, can be used as a marker or detector of a particular immune system disease or disorder.

Follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, may be useful in treating or detecting deficiencies or disorders of hematopoietic cells. Follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, could be used to increase differentiation and proliferation of hematopoietic cells, including the pluripotent stem cells, in an effort to treat those disorders associated with a decrease in certain (or many) types hematopoietic cells. Examples of immunologic deficiency syndromes include, but are not limited to: blood protein disorders (e.g. agammaglobulinemia, dysgammaglobulinemia), ataxia telangiectasia, common variable immunodeficiency, Digeorge Syndrome, HIV infection, HTLV-BLV infection, leukocyte adhesion deficiency syndrome, lymphopenia, phagocyte bactericidal dysfunction, severe combined immunodeficiency (SCIDs), Wiskott-Aldrich Disorder, anemia, thrombocytopenia, or hemoglobinuria.

Moreover, follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, can also be used to modulate hemostatic (the stopping of bleeding) or thrombolytic activity (clot formation). For example, by increasing hemostatic or thrombolytic activity, follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, could be used to treat blood coagulation disorders (e.g., afibrinogenemia, factor deficiencies), blood platelet disorders (e.g. thrombocytopenia), or wounds resulting from trauma, surgery, or other causes. Alternatively, follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of

follistatin-3, that can decrease hemostatic or thrombolytic activity could be used to inhibit or dissolve clotting, important in the treatment of heart attacks (infarction), strokes, or scarring.

Follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, may also be useful in treating or detecting autoimmune disorders. Many autoimmune disorders result from inappropriate recognition of self as foreign material by immune cells. This inappropriate recognition results in an immune response leading to the destruction of the host tissue. Therefore, the administration of follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, that can inhibit an immune response, particularly the proliferation, differentiation, or chemotaxis of T-cells, may be an effective therapy in preventing autoimmune disorders.

Examples of autoimmune disorders that can be treated or detected include, but are not limited to: Addison's Disease, hemolytic anemia, antiphospholipid syndrome, rheumatoid arthritis, dermatitis, allergic encephalomyelitis, glomerulonephritis, Goodpasture's Syndrome, Graves' Disease, Multiple Sclerosis, Myasthenia Gravis, Neuritis, Ophthalmia, Bullous Pemphigoid, Pemphigus, Polyendocrinopathies, Purpura, Reiter's Disease, Stiff-Man Syndrome, Autoimmune Thyroiditis, Systemic Lupus Erythematosus, Autoimmune Pulmonary Inflammation, Guillain-Barre Syndrome, insulin dependent diabetes mellitis, and autoimmune inflammatory eye disease.

Similarly, allergic reactions and conditions, such as asthma (particularly allergic asthma) or other respiratory problems, may also be treated by follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3. Moreover, these molecules can be used to treat anaphylaxis, hypersensitivity to an antigenic molecule, or blood group incompatibility.

Follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, may also be used to treat and/or prevent organ rejection or graft-versus-host disease (GVHD). Organ rejection occurs by host immune cell destruction of the transplanted tissue through an immune response. Similarly, an immune response is also involved in GVHD, but, in this case, the foreign transplanted immune cells destroy the host tissues. The administration of follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, that inhibits an immune response, particularly the

proliferation, differentiation, or chemotaxis of T-cells, may be an effective therapy in preventing organ rejection or GVHD.

Similarly, follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, may also be used to modulate inflammation. For example, follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, may inhibit the proliferation and differentiation of cells involved in an inflammatory response. These molecules can be used to treat inflammatory conditions, both chronic and acute conditions, including inflammation associated with infection (e.g., septic shock, sepsis, or systemic inflammatory response syndrome (SIRS)), ischemia-reperfusion injury, endotoxin lethality, arthritis, complement-mediated hyperacute rejection, nephritis, cytokine or chemokine induced lung injury, inflammatory bowel disease, Crohn's disease, or resulting from over production of cytokines (e.g., TNF or IL-1.)

Hyperproliferative Disorders.

Follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, can be used to treat or detect hyperproliferative disorders, including neoplasms. Follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, may inhibit the proliferation of the disorder through direct or indirect interactions. Alternatively, follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, may proliferate other cells which can inhibit the hyperproliferative disorder.

For example, by increasing an immune response, particularly increasing antigenic qualities of the hyperproliferative disorder or by proliferating, differentiating, or mobilizing T-cells, hyperproliferative disorders can be treated. This immune response may be increased by either enhancing an existing immune response, or by initiating a new immune response. Alternatively, decreasing an immune response may also be a method of treating hyperproliferative disorders, such as a chemotherapeutic agent.

Examples of hyperproliferative disorders that can be treated or detected by follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, include, but are not limited to neoplasms located in the: abdomen, bone, breast, digestive system, liver, pancreas, peritoneum, endocrine glands (adrenal, parathyroid, pituitary,

testicles, ovary, thymus, thyroid), eye, head and neck, nervous (central and peripheral), lymphatic system, pelvic, skin, soft tissue, spleen, thoracic, and urogenital.

Similarly, other hyperproliferative disorders can also be treated or detected by follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3. Examples of such hyperproliferative disorders include, but are not limited to: hypergammaglobulinemia, lymphoproliferative disorders, paraproteinemias, purpura, sarcoidosis, Sezary Syndrome, Waldenstron's Macroglobulinemia, Gaucher's Disease, histiocytosis, and any other hyperproliferative disease, besides neoplasia, located in an organ system listed above.

Cardiovascular Disorders.

Follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, encoding follistatin-3 may be used to treat cardiovascular disorders, including peripheral artery disease, such as limb ischemia.

Cardiovascular disorders include cardiovascular abnormalities, such as arterio-arterial fistula, arteriovenous fistula, cerebral arteriovenous malformations, congenital heart defects, pulmonary atresia, and Scimitar Syndrome. Congenital heart defects include aortic coarctation, cor triatriatum, coronary vessel anomalies, crisscross heart, dextrocardia, patent ductus arteriosus, Ebstein's anomaly, Eisenmenger complex, hypoplastic left heart syndrome, levocardia, tetralogy of fallot, transposition of great vessels, double outlet right ventricle, tricuspid atresia, persistent truncus arteriosus, and heart septal defects, such as aortopulmonary septal defect, endocardial cushion defects, Lutembacher's Syndrome, trilogy of Fallot, ventricular heart septal defects.

Cardiovascular disorders also include heart disease, such as arrhythmias, carcinoid heart disease, high cardiac output, low cardiac output, cardiac tamponade, endocarditis (including bacterial), heart aneurysm, cardiac arrest, congestive heart failure, congestive cardiomyopathy, paroxysmal dyspnea, cardiac edema, heart hypertrophy, congestive cardiomyopathy, left ventricular hypertrophy, right ventricular hypertrophy, post-infarction heart rupture, ventricular septal rupture, heart valve diseases, myocardial diseases, myocardial ischemia, pericardial effusion, pericarditis (including constrictive and tuberculous), pneumopericardium, postpericardiotomy

syndrome, pulmonary heart disease, rheumatic heart disease, ventricular dysfunction, hyperemia, cardiovascular pregnancy complications, Scimitar Syndrome, cardiovascular syphilis, and cardiovascular tuberculosis.

Arrhythmias include sinus arrhythmia, atrial fibrillation, atrial flutter, bradycardia, extrasystole, Adams-Stokes Syndrome, bundle-branch block, sinoatrial block, long QT syndrome, parasystole, Lown-Ganong-Levine Syndrome, Mahaim-type pre-excitation syndrome, Wolff-Parkinson-White syndrome, sick sinus syndrome, tachycardias, and ventricular fibrillation. Tachycardias include paroxysmal tachycardia, supraventricular tachycardia, accelerated idioventricular rhythm, atrioventricular nodal reentry tachycardia, ectopic atrial tachycardia, ectopic junctional tachycardia, sinoatrial nodal reentry tachycardia, sinus tachycardia, Torsades de Pointes, and ventricular tachycardia.

Heart valve disease include aortic valve insufficiency, aortic valve stenosis, hear murmurs, aortic valve prolapse, mitral valve prolapse, tricuspid valve prolapse, mitral valve insufficiency, mitral valve stenosis, pulmonary atresia, pulmonary valve insufficiency, pulmonary valve stenosis, tricuspid atresia, tricuspid valve insufficiency, and tricuspid valve stenosis.

Myocardial diseases include alcoholic cardiomyopathy, congestive cardiomyopathy, hypertrophic cardiomyopathy, aortic subvalvular stenosis, pulmonary subvalvular stenosis, restrictive cardiomyopathy, Chagas cardiomyopathy, endocardial fibroelastosis, endomyocardial fibrosis, Kearns Syndrome, myocardial reperfusion injury, and myocarditis.

Myocardial ischemias include coronary disease, such as angina pectoris, coronary aneurysm, coronary arteriosclerosis, coronary thrombosis, coronary vasospasm, myocardial infarction and myocardial stunning.

Cardiovascular diseases also include vascular diseases such as aneurysms, angiodysplasia, angiomatosis, bacillary angiomatosis, Hippel-Lindau Disease, Klippel-Trenaunay-Weber Syndrome, Sturge-Weber Syndrome, angioneurotic edema, aortic diseases, Takayasu's Arteritis, aortitis, Leriche's Syndrome, arterial occlusive diseases, arteritis, enarteritis, polyarteritis nodosa, cerebrovascular disorders, diabetic angiopathies, diabetic retinopathy, embolisms, thrombosis, erythromelalgia, hemorrhoids, hepatic veno-occlusive disease, hypertension, hypotension, ischemia, peripheral vascular

diseases, phlebitis, pulmonary veno-occlusive disease, Raynaud's disease, CREST syndrome, retinal vein occlusion, Scimitar syndrome, superior vena cava syndrome, telangiectasia, atacia telangiectasia, hereditary hemorrhagic telangiectasia, varicocele, varicose veins, varicose ulcer, vasculitis, and venous insufficiency.

Aneurysms include dissecting aneurysms, false aneurysms, infected aneurysms, ruptured aneurysms, aortic aneurysms, cerebral aneurysms, coronary aneurysms, heart aneurysms, and iliac aneurysms.

Arterial occlusive diseases include arteriosclerosis, intermittent claudication, carotid stenosis, fibromuscular dysplasias, mesenteric vascular occlusion, Moyamoya disease, renal artery obstruction, retinal artery occlusion, and thromboangiitis obliterans.

Cerebrovascular disorders include carotid artery diseases, cerebral amyloid angiopathy, cerebral aneurysm, cerebral anoxia, cerebral arteriosclerosis, cerebral arteriovenous malformation, cerebral artery diseases, cerebral embolism and thrombosis, carotid artery thrombosis, sinus thrombosis, Wallenberg's syndrome, cerebral hemorrhage, epidural hematoma, subdural hematoma, subaraxhnoid hemorrhage, cerebral infarction, cerebral ischemia (including transient), subclavian steal syndrome, periventricular leukomalacia, vascular headache, cluster headache, migraine, and vertebrobasilar insufficiency.

Embolisms include air embolisms, amniotic fluid embolisms, cholesterol embolisms, blue toe syndrome, fat embolisms, pulmonary embolisms, and thromoboembolisms. Thrombosis include coronary thrombosis, hepatic vein thrombosis, retinal vein occlusion, carotid artery thrombosis, sinus thrombosis, Wallenberg's syndrome, and thrombophlebitis.

Ischemia includes cerebral ischemia, ischemic colitis, compartment syndromes, anterior compartment syndrome, myocardial ischemia, reperfusion injuries, and peripheral limb ischemia. Vasculitis includes aortitis, arteritis, Behcet's Syndrome, Churg-Strauss Syndrome, mucocutaneous lymph node syndrome, thromboangiitis obliterans, hypersensitivity vasculitis, Schoenlein-Henoch purpura, allergic cutaneous vasculitis, and Wegener's granulomatosis.

Follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, are especially effective for the treatment of critical limb ischemia and

coronary disease. As shown in the Examples, administration of follistatin-3 polynucleotides and polypeptides to an experimentally induced ischemia rabbit hindlimb may restore blood pressure ratio, blood flow, angiographic score, and capillary density.

Follistatin-3 polypeptides may be administered using any method known in the art, including, but not limited to, direct needle injection at the delivery site, intravenous injection, topical administration, catheter infusion, biolistic injectors, particle accelerators, gelfoam sponge depots, other commercially available depot materials, osmotic pumps, oral or suppositorial solid pharmaceutical formulations, decanting or topical applications during surgery, aerosol delivery. Such methods are known in the art. Follistatin-3 polypeptides may be administered as part of a pharmaceutical composition, described in more detail below. Methods of delivering follistatin-3 polynucleotides are described in more detail herein.

Angiogenesis.

The naturally occurring balance between endogenous stimulators and inhibitors of angiogenesis is one in which inhibitory influences predominate. Rastinejad *et al., Cell 56*:345-355 (1989). In those rare instances in which neovascularization occurs under normal physiological conditions, such as wound healing, organ regeneration, embryonic development, and female reproductive processes, angiogenesis is stringently regulated and spatially and temporally delimited. Under conditions of pathological angiogenesis such as that characterizing solid tumor growth, these regulatory controls fail. Unregulated angiogenesis becomes pathologic and sustains progression of many neoplastic and non-neoplastic diseases. A number of serious diseases are dominated by abnormal neovascularization including solid tumor growth and metastases, arthritis, some types of eye disorders, and psoriasis. See, e.g., reviews by Moses *et al., Biotech. 9*:630-634 (1991); Folkman *et al., N. Engl. J. Med., 333*:1757-1763 (1995); Auerbach *et al., J. Microvasc. Res. 29*:401-411 (1985); Folkman, Advances in Cancer Research, eds. Klein and Weinhouse, Academic Press, New York, pp. 175-203 (1985); Patz, *Am. J. Opthalmol. 94*:715-743 (1982); and Folkman *et al.*, Science *221*:719-725 (1983). In a number of pathological conditions, the process of angiogenesis contributes to the disease state. For example, significant data have accumulated which suggest that the growth of

solid tumors is dependent on angiogenesis. Folkman and Klagsbrun, *Science 235*:442-447 (1987).

The present invention provides for treatment of diseases or disorders associated with neovascularization by administration of the follistatin-3 polynucleotides and/or polypeptides of the invention, as well as agonists or antagonists of follistatin-3. Malignant and metastatic conditions which can be treated with the polynucleotides and polypeptides, or agonists or antagonists of the invention include, but are not limited to, malignancies, solid tumors, and cancers described herein and otherwise known in the art (for a review of such disorders, see Fishman *et al.*, Medicine, 2d Ed., J. B. Lippincott Co., Philadelphia (1985)).

Ocular disorders associated with neovascularization which can be treated with the follistatin-3 polynucleotides and polypeptides of the present invention (including follistatin-3 agonists and/or antagonists) include, but are not limited to: neovascular glaucoma, diabetic retinopathy, retinoblastoma, retrolental fibroplasia, uveitis, retinopathy of prematurity macular degeneration, corneal graft neovascularization, as well as other eye inflammatory diseases, ocular tumors and diseases associated with choroidal or iris neovascularization. See, e.g., reviews by Waltman *et al.*, *Am. J. Ophthal. 85*:704-710 (1978) and Gartner *et al.*, *Surv. Ophthal. 22*:291-312 (1978).

Additionally, disorders which can be treated with the follistatin-3 polynucleotides and polypeptides of the present invention (including follistatin-3 agonist and/or antagonists) include, but are not limited to, hemangioma, arthritis, psoriasis, angiofibroma, atherosclerotic plaques, delayed wound healing, granulations, hemophilic joints, hypertrophic scars, nonunion fractures, Osler-Weber syndrome, pyogenic granuloma, scleroderma, trachoma, and vascular adhesions.

Moreover, disorders and/or states, which can be treated with be treated with the follistatin-3 polynucleotides and polypeptides of the present invention (including follistatin-3 agonist and/or antagonists) include, but are not limited to, solid tumors, blood born tumors such as leukemias, tumor metastasis, Kaposi's sarcoma, benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas, rheumatoid arthritis, psoriasis, ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal

graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis, retinoblastoma, and uvietis, delayed wound healing, endometriosis, vasculogenesis, granulations, hypertrophic scars (keloids), nonunion fractures, scleroderma, trachoma, vascular adhesions, myocardial angiogenesis, coronary collaterals, cerebral collaterals, arteriovenous malformations, ischemic limb angiogenesis, Osler-Webber Syndrome, plaque neovascularization, telangiectasia, hemophiliac joints, angiofibroma fibromuscular dysplasia, wound granulation, Crohn's disease, atherosclerosis, birth control agent by preventing vascularization required for embryo implantation controlling menstruation, diseases that have angiogenesis as a pathologic consequence such as cat scratch disease (Rochele minalia quintosa), ulcers (Helicobacter pylori), Bartonellosis and bacillary angiomatosis.

Diseases at the Cellular Level.

Diseases associated with increased cell survival or the inhibition of apoptosis that could be treated or detected by follistatin-3 polynucleotides or polypeptides, as well as antagonists or agonists of follistatin-3, include cancers (such as follicular lymphomas, carcinomas with p53 mutations, and hormone-dependent tumors, including, but not limited to colon cancer, cardiac tumors, pancreatic cancer, melanoma, retinoblastoma, glioblastoma, lung cancer, intestinal cancer, testicular cancer, stomach cancer, neuroblastoma, myxoma, myoma, lymphoma, endothelioma, osteoblastoma, osteoclastoma, osteosarcoma, chondrosarcoma, adenoma, breast cancer, prostate cancer, Kaposi's sarcoma and ovarian cancer); autoimmune disorders (such as, multiple sclerosis, Sjogren's syndrome, Hashimoto's thyroiditis, biliary cirrhosis, Behcet's disease, Crohn's disease, polymyositis, systemic lupus erythematosus and immune-related glomerulonephritis and rheumatoid arthritis) and viral infections (such as herpes viruses, pox viruses and adenoviruses), inflammation, graft v. host disease, acute graft rejection, and chronic graft rejection. In preferred embodiments, follistatin-3 polynucleotides, polypeptides, and/or antagonists of the invention are used to inhibit growth, progression, and/or metasis of cancers, in particular those listed above.

Additional diseases or conditions associated with increased cell survival that could be treated or detected by follistatin-3 polynucleotides or polypeptides, or agonists

or antagonists of follistatin-3, include, but are not limited to, progression, and/or metastases of malignancies and related disorders such as leukemia (including acute leukemias (e.g., acute lymphocytic leukemia, acute myelocytic leukemia (including myeloblastic, promyelocytic, myelomonocytic, monocytic, and erythroleukemia)) and chronic leukemias (e.g., chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia)), polycythemia vera, lymphomas (e.g., Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, and solid tumors including, but not limited to, sarcomas and carcinomas such as fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, melanoma, neuroblastoma, and retinoblastoma.

Diseases associated with increased apoptosis that could be treated or detected by follistatin-3 polynucleotides or polypeptides, as well as agonists or antagonists of follistatin-3, include AIDS; neurodegenerative disorders (such as Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis, Retinitis pigmentosa, Cerebellar degeneration and brain tumor or prior associated disease); autoimmune disorders (such as, multiple sclerosis, Sjogren's syndrome, Hashimoto's thyroiditis, biliary cirrhosis, Behcet's disease, Crohn's disease, polymyositis, systemic lupus erythematosus and immune-related glomerulonephritis and rheumatoid arthritis) myelodysplastic syndromes (such as aplastic anemia), graft v. host disease, ischemic injury (such as that caused by myocardial infarction, stroke and reperfusion injury), liver injury (e.g., hepatitis related

liver injury, ischemia/reperfusion injury, cholestosis (bile duct injury) and liver cancer); toxin-induced liver disease (such as that caused by alcohol), septic shock, cachexia and anorexia.

Wound Healing and Epithelial Cell Proliferation.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing follistatin-3 polynucleotides or polypeptides, as well as agonists or antagonists of follistatin-3, for therapeutic purposes, for example, to stimulate epithelial cell proliferation and basal keratinocytes for the purpose of wound healing, and to stimulate hair follicle production and healing of dermal wounds. follistatin-3 polynucleotides or polypeptides, as well as agonists or antagonists of follistatin-3, may be clinically useful in stimulating wound healing including surgical wounds, excisional wounds, deep wounds involving damage of the dermis and epidermis, eye tissue wounds, dental tissue wounds, oral cavity wounds, diabetic ulcers, dermal ulcers, cubitus ulcers, arterial ulcers, venous stasis ulcers, burns resulting from heat exposure or chemicals, and other abnormal wound healing conditions such as uremia, malnutrition, vitamin deficiencies and complications associted with systemic treatment with steroids, radiation therapy and antineoplastic drugs and antimetabolites. Follistatin-3 polynucleotides or polypeptides, as well as agonists or antagonists of follistatin-3, could be used to promote dermal reestablishment subsequent to dermal loss

Follistatin-3 polynucleotides or polypeptides, as well as agonists or antagonists of follistatin-3, could be used to increase the adherence of skin grafts to a wound bed and to stimulate re-epithelialization from the wound bed. The following are types of grafts that follistatin-3 polynucleotides or polypeptides, agonists or antagonists of follistatin-3, could be used to increase adherence to a wound bed: autografts, artificial skin, allografts, autodermic graft, autoepdermic grafts, avacular grafts, Blair-Brown grafts, bone graft, brephoplastic grafts, cutis graft, delayed graft, dermic graft, epidermic graft, fascia graft, full thickness graft, heterologous graft, xenograft, homologous graft, hyperplastic graft, lamellar graft, mesh graft, mucosal graft, Ollier-Thiersch graft, onenpal graft, patch graft, pedicle graft, penetrating graft, split skin graft, thick split graft. follistatin-3

polynucleotides or polypeptides, as well as agonists or antagonists of follistatin-3, can be used to promote skin strength and to improve the appearance of aged skin.

It is believed that follistatin-3 polynucleotides or polypeptides, as well as agonists or antagonists of follistatin-3, will also produce changes in hepatocyte proliferation, and epithelial cell proliferation in the lung, breast, pancreas, stomach, small intestine, and large intestine. Follistatin-3 polynucleotides or polypeptides, as well as agonists or antagonists of follistatin-3, could promote proliferation of epithelial cells such as sebocytes, hair follicles, hepatocytes, type II pneumocytes, mucin-producing goblet cells, and other epithelial cells and their progenitors contained within the skin, lung, liver, and gastrointestinal tract. Follistatin-3 polynucleotides or polypeptides, agonists or antagonists of follistatin-3, may promote proliferation of endothelial cells, keratinocytes, and basal keratinocytes.

Follistatin-3 polynucleotides or polypeptides, as well as agonists or antagonists of follistatin-3, could also be used to reduce the side effects of gut toxicity that result from radiation, chemotherapy treatments or viral infections. Follistatin-3 polynucleotides or polypeptides, as well as agonists or antagonists of follistatin-3, may have a cytoprotective effect on the small intestine mucosa. Follistatin-3 polynucleotides or polypeptides, as well as agonists or antagonists of follistatin-3, may also stimulate healing of mucositis (mouth ulcers) that result from chemotherapy and viral infections.

Follistatin-3 polynucleotides or polypeptides, as well as agonists or antagonists of follistatin-3, could further be used in full regeneration of skin in full and partial thickness skin defects, including burns, (i.e., repopulation of hair follicles, sweat glands, and sebaceous glands), treatment of other skin defects such as psoriasis. Follistatin-3 polynucleotides or polypeptides, as well as agonists or antagonists of follistatin-3, could be used to treat epidermolysis bullosa, a defect in adherence of the epidermis to the underlying dermis which results in frequent, open and painful blisters by accelerating reepithelialization of these lesions. Follistatin-3 polynucleotides or polypeptides, as well as agonists or antagonists of follistatin-3, could also be used to treat gastric and doudenal ulcers and help heal by scar formation of the mucosal lining and regeneration of glandular mucosa and duodenal mucosal lining more rapidly. Inflamamatory bowel diseases, such as Crohn's disease and ulcerative colitis, are diseases which result in

destruction of the mucosal surface of the small or large intestine, respectively. Thus, follistatin-3 polynucleotides or polypeptides, as well as agonists or antagonists of follistatin-3, could be used to promote the resurfacing of the mucosal surface to aid more rapid healing and to prevent progression of inflammatory bowel disease. Treatment with follistatin-3 polynucleotides or polypeptides, agonists or antagonists of follistatin-3, is expected to have a significant effect on the production of mucus throughout the gastrointestinal tract and could be used to protect the intestinal mucosa from injurious substances that are ingested or following surgery. Follistatin-3 polynucleotides or polypeptides, as well as agonists or antagonists of follistatin-3, could be used to treat diseases associate with the under expression of follistatin-3.

Moreover, follistatin-3 polynucleotides or polypeptides, as well as agonists or antagonists of follistatin-3, could be used to prevent and heal damage to the lungs due to various pathological states. A growth factor such as follistatin-3 polynucleotides or polypeptides, as well as agonists or antagonists of follistatin-3, which could stimulate proliferation and differentiation and promote the repair of alveoli and brochiolar epithelium to prevent or treat acute or chronic lung damage. For example, emphysema, which results in the progressive loss of aveoli, and inhalation injuries, i.e., resulting from smoke inhalation and burns, that cause necrosis of the bronchiolar epithelium and alveoli could be effectively treated using follistatin-3 polynucleotides or polypeptides, agonists or antagonists of follistatin-3. Also, follistatin-3 polynucleotides or polypeptides, as well as agonists or antagonists of follistatin-3, could be used to stimulate the proliferation of and differentiation of type II pneumocytes, which may help treat or prevent disease such as hyaline membrane diseases, such as infant respiratory distress syndrome and bronchopulmonary displasia, in premature infants.

Follistatin-3 polynucleotides or polypeptides, as well as agonists or antagonists of follistatin-3, could stimulate the proliferation and differentiation of hepatocytes and, thus, could be used to alleviate or treat liver diseases and pathologies such as fulminant liver failure caused by cirrhosis, liver damage caused by viral hepatitis and toxic substances (i.e., acetaminophen, carbon tetraholoride and other hepatotoxins known in the art).

In addition, follistatin-3 polynucleotides or polypeptides, as well as agonists or antagonists of follistatin-3, could be used treat or prevent the onset of diabetes mellitus.

In patients with newly diagnosed Types I and II diabetes, where some islet cell function remains, follistatin-3 polynucleotides or polypeptides, as well as agonists or antagonists of follistatin-3, could be used to maintain the islet function so as to alleviate, delay or prevent permanent manifestation of the disease. Also, follistatin-3 polynucleotides or polypeptides, as well as agonists or antagonists of follistatin-3, could be used as an auxiliary in islet cell transplantation to improve or promote islet cell function.

Infectious Disease.

Follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, can be used to treat or detect infectious agents. For example, by increasing the immune response, particularly increasing the proliferation and differentiation of B and/or T cells, infectious diseases may be treated. The immune response may be increased by either enhancing an existing immune response, or by initiating a new immune response. Alternatively, follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, may also directly inhibit the infectious agent, without necessarily eliciting an immune response.

Viruses are one example of an infectious agent that can cause disease or symptoms that can be treated or detected by follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3. Examples of viruses, include, but are not limited to the following DNA and RNA viral families: Arbovirus, Adenoviridae, Arenaviridae, Arterivirus, Birnaviridae, Bunyaviridae, Caliciviridae, Circoviridae, Coronaviridae, Flaviviridae, Hepadnaviridae (Hepatitis), Herpesviridae (such as, Cytomegalovirus, Herpes Simplex, Herpes Zoster), Mononegavirus (e.g., Paramyxoviridae, Morbillivirus, Rhabdoviridae), Orthomyxoviridae (e.g., Influenza), Papovaviridae, Parvoviridae, Picornaviridae, Poxviridae (such as Smallpox or Vaccinia), Reoviridae (e.g., Rotavirus), Retroviridae (HTLV-I, HTLV-II, Lentivirus), and Togaviridae (e.g., Rubivirus). Viruses falling within these families can cause a variety of diseases or symptoms, including, but not limited to: arthritis, bronchiolitis, encephalitis, eye infections (e.g., conjunctivitis, keratitis), chronic fatigue syndrome, hepatitis (A, B, C, E, Chronic Active, Delta), meningitis, opportunistic infections (e.g., AIDS), pneumonia, Burkitt's Lymphoma, chickenpox , hemorrhagic fever, Measles, Mumps,

Parainfluenza, Rabies, the common cold, Polio, leukemia, Rubella, sexually transmitted diseases, skin diseases (e.g., Kaposi's, warts), and viremia. Follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, can be used to treat or detect any of these symptoms or diseases.

Similarly, bacterial or fungal agents that can cause disease or symptoms and that can be treated or detected by follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, include, but not limited to, the following Gram-Negative and Gram-positive bacterial families and fungi: Actinomycetales (e.g., Corynebacterium, Mycobacterium, Norcardia), Aspergillosis, Bacillaceae (e.g., Anthrax, Clostridium), Bacteroidaceae, Blastomycosis, Bordetella, Borrelia, Brucellosis, Candidiasis, Campylobacter, Coccidioidomycosis, Cryptococcosis, Dermatocycoses, Enterobacteriaceae (Klebsiella, Salmonella, Serratia, Yersinia), Erysipelothrix, Helicobacter, Legionellosis, Leptospirosis, Listeria, Mycoplasmatales, Neisseriaceae (e.g., Acinetobacter, Gonorrhea, Menigococcal), Pasteurellacea Infections (e.g., Actinobacillus, Heamophilus, Pasteurella), Pseudomonas, Rickettsiaceae, Chlamydiaceae, Syphilis, and Staphylococcal. These bacterial or fungal families can cause the following diseases or symptoms, including, but not limited to: bacteremia, endocarditis, eye infections (conjunctivitis, tuberculosis, uveitis), gingivitis, opportunistic infections (e.g., AIDS related infections), paronychia, prosthesis-related infections, Reiter's Disease, respiratory tract infections, such as Whooping Cough or Empyema, sepsis, Lyme Disease, Cat-Scratch Disease, Dysentery, Paratyphoid Fever, food poisoning, Typhoid, pneumonia, Gonorrhea, meningitis, Chlamydia, Syphilis, Diphtheria, Leprosy, Paratuberculosis, Tuberculosis, Lupus, Botulism, gangrene, tetanus, impetigo, Rheumatic Fever, Scarlet Fever, sexually transmitted diseases, skin diseases (e.g., cellulitis, dermatocycoses), toxemia, urinary tract infections, wound infections. Follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, can be used to treat or detect any of these symptoms or diseases.

Moreover, parasitic agents causing disease or symptoms that can be treated or detected by follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, include, but not limited to, the following families: Amebiasis, Babesiosis, Coccidiosis, Cryptosporidiosis, Dientamoebiasis, Dourine, Ectoparasitic, Giardiasis,

Helminthiasis, Leishmaniasis, Theileriasis, Toxoplasmosis, Trypanosomiasis, and Trichomonas. These parasites can cause a variety of diseases or symptoms, including, but not limited to: Scabies, Trombiculiasis, eye infections, intestinal disease (e.g., dysentery, giardiasis), liver disease, lung disease, opportunistic infections (e.g., AIDS related), Malaria, pregnancy complications, and toxoplasmosis. Follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, can be used to treat or detect any of these symptoms or diseases.

Preferably, treatment using follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, could either be by administering an effective amount of follistatin-3 polypeptide to the patient, or by removing cells from the patient, supplying the cells with follistatin-3 polynucleoside, and returning the engineered cells to the patient (ex vivo therapy). Moreover, the follistatin-3 polypeptide or polynucleotide can be used as an antigen in a vaccine to raise an immune response against infectious disease.

Regeneration.

Follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, can be used to differentiate, proliferate, and attract cells, leading to the regeneration of tissues. (See, Science 276:59-87 (1997).) The regeneration of tissues could be used to repair, replace, or protect tissue damaged by congenital defects, trauma (wounds, burns, incisions, or ulcers), age, disease (e.g. osteoporosis, osteocarthritis, periodontal disease, liver failure), surgery, including cosmetic plastic surgery, fibrosis, reperfusion injury, or systemic cytokine damage.

Tissues that could be regenerated using the present invention include organs (e.g., pancreas, liver, intestine, kidney, skin, endothelium), muscle (smooth, skeletal or cardiac), vasculature (including vascular and lymphatics), nervous, hematopoietic, and skeletal (bone, cartilage, tendon, and ligament) tissue. Preferably, regeneration occurs without or decreased scarring. Regeneration also may include angiogenesis.

Moreover, follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, may increase regeneration of tissues difficult to heal. For example, increased tendon/ligament regeneration would quicken recovery time after

damage. Follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3,of the present invention could also be used prophylactically in an effort to avoid damage. Specific diseases that could be treated include of tendinitis, carpal tunnel syndrome, and other tendon or ligament defects. A further example of tissue regeneration of non-healing wounds includes pressure ulcers, ulcers associated with vascular insufficiency, surgical, and traumatic wounds.

Similarly, nerve and brain tissue could also be regenerated by using follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, to proliferate and differentiate nerve cells. Diseases that could be treated using this method include central and peripheral nervous system diseases, neuropathies, or mechanical and traumatic disorders (e.g., spinal cord disorders, head trauma, cerebrovascular disease, and stoke). Specifically, diseases associated with peripheral nerve injuries, peripheral neuropathy (e.g., resulting from chemotherapy or other medical therapies), localized neuropathies, and central nervous system diseases (e.g., Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and Shy-Drager syndrome), could all be treated using the follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3.

Chemotaxis.

Follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, may have chemotaxis activity. A chemotaxic molecule attracts or mobilizes cells (e.g., monocytes, fibroblasts, neutrophils, T-cells, mast cells, eosinophils, epithelial and/or endothelial cells) to a particular site in the body, such as inflammation, infection, or site of hyperproliferation. The mobilized cells can then fight off and/or heal the particular trauma or abnormality.

Follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, may increase chemotaxic activity of particular cells. These chemotactic molecules can then be used to treat inflammation, infection, hyperproliferative disorders, or any immune system disorder by increasing the number of cells targeted to a particular location in the body. For example, chemotaxic molecules can be used to treat wounds and other trauma to tissues by attracting immune cells to the injured location. As a

chemotactic molecule, follistatin-3 could also attract fibroblasts, which can be used to treat wounds.

It is also contemplated that follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, may inhibit chemotactic activity. These molecules could also be used to treat disorders. Thus, follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, could be used as an inhibitor of chemotaxis.

Binding Activity.

Follistatin-3 polypeptides may be used to screen for molecules that bind to follistatin-3 or for molecules to which follistatin-3 binds. The binding of follistatin-3 and the molecule may activate (agonist), increase, inhibit (antagonist), or decrease activity of the follistatin-3 or the molecule bound. Examples of such molecules include antibodies, oligonucleotides, proteins (e.g., receptors),or small molecules.

Preferably, the molecule is closely related to the natural ligand of follistatin-3, e.g., a fragment of the ligand, or a natural substrate, a ligand, a structural or functional mimetic. (See, Coligan et al., Current Protocols in Immunology 1(2):Chapter 5 (1991).) Similarly, the molecule can be closely related to the natural receptor to which follistatin-3 binds, or at least, a fragment of the receptor capable of being bound by follistatin-3 (e.g., active site). In either case, the molecule can be rationally designed using known techniques.

Preferably, the screening for these molecules involves producing appropriate cells which express follistatin-3, either as a secreted protein or on the cell membrane. Preferred cells include cells from mammals, yeast, Drosophila, or E. coli. Cells expressing follistatin-3 (or cell membrane containing the expressed polypeptide) are then preferably contacted with a test compound potentially containing the molecule to observe binding, stimulation, or inhibition of activity of either follistatin-3 or the molecule.

The assay may simply test binding of a candidate compound to follistatin-3, wherein binding is detected by a label, or in an assay involving competition with a labeled competitor. Further, the assay may test whether the candidate compound results in a signal generated by binding to follistatin-3.

Alternatively, the assay can be carried out using cell-free preparations, polypeptide/molecule affixed to a solid support, chemical libraries, or natural product mixtures. The assay may also simply comprise the steps of mixing a candidate compound with a solution containing follistatin-3, measuring follistatin-3/molecule activity or binding, and comparing the follistatin-3/molecule activity or binding to a standard.

Preferably, an ELISA assay can measure follistatin-3 level or activity in a sample (e.g., biological sample) using a monoclonal or polyclonal antibody. The antibody can measure follistatin-3 level or activity by either binding, directly or indirectly, to follistatin-3 or by competing with follistatin-3 for a substrate.

Additionally, the receptor to which follistatin-3 binds can be identified by numerous methods known to those of skill in the art, for example, ligand panning and FACS sorting (Coligan, et al., Current Protocols in Immun., 1(2), Chapter 5, (1991)). For example, expression cloning is employed wherein polyadenylated RNA is prepared from a cell responsive to the polypeptides, for example, NIH3T3 cells which are known to contain multiple receptors for the FGF family proteins, and SC-3 cells, and a cDNA library created from this RNA is divided into pools and used to transfect COS cells or other cells that are not responsive to the polypeptides. Transfected cells which are grown on glass slides are exposed to the polypeptide of the present invention, after they have been labelled. The polypeptides can be labeled by a variety of means including iodination or inclusion of a recognition site for a site-specific protein kinase.

Following fixation and incubation, the slides are subjected to auto-radiographic analysis. Positive pools are identified and sub-pools are prepared and re-transfected using an iterative sub-pooling and re-screening process, eventually yielding a single clones that encodes the putative receptor.

As an alternative approach for receptor identification, the labeled polypeptides can be photoaffinity linked with cell membrane or extract preparations that express the receptor molecule. Cross-linked material is resolved by PAGE analysis and exposed to X-ray film. The labeled complex containing the receptors of the polypeptides can be excised, resolved into peptide fragments, and subjected to protein microsequencing. The amino acid sequence obtained from microsequencing would be used to design a set of

degenerate oligonucleotide probes to screen a cDNA library to identify the genes encoding the putative receptors.

Moreover, the techniques of gene-shuffling, motif-shuffling, exon-shuffling, and/or codon-shuffling (collectively referred to as "DNA shuffling") may be employed to modulate the activities of follistatin-3 thereby effectively generating agonists and antagonists of follistatin-3. *See generally*, U.S. Patent Nos. 5,605,793, 5,811,238, 5,830,721, 5,834,252, and 5,837,458, and Patten, P. A., *et al.*, *Curr. Opinion Biotechnol.* 8:724-33 (1997); Harayama, S. *Trends Biotechnol.* 16(2):76-82 (1998); Hansson, L. O., *et al.*, *J. Mol. Biol.* 287:265-76 (1999); and Lorenzo, M. M. and Blasco, R. *Biotechniques* 24(2):308-13 (1998) (each of these patents and publications are hereby incorporated by reference). In one embodiment, alteration of follistatin-3 polynucleotides and corresponding polypeptides may be achieved by DNA shuffling. DNA shuffling involves the assembly of two or more DNA segments into a desired follistatin-3 molecule by homologous, or site-specific, recombination. In another embodiment, follistatin-3 polynucleotides and corresponding polypeptides may be altered by being subjected to random mutagenesis by error-prone PCR, random nucleotide insertion or other methods prior to recombination. In another embodiment, one or more components, motifs, sections, parts, domains, fragments, etc., of follistatin-3 may be recombined with one or more components, motifs, sections, parts, domains, fragments, etc. of one or more heterologous molecules. In preferred embodiments, the heterologous molecules are follistatin-3 family members. In further preferred embodiments, the heterologous molecule is a growth factor such as, for example, plateles-derived growth factor (PDGF), insulin-like growth factor (IGF-I), transforming growth factor (TGF)-alpha, epidermal growth factor (EGF), fibroblast growth factor (FGF), TGF-beta, bone morphogenetic protein (BMP)-2, BMP-4, BMP-5, BMP-6, BMP-7, activins A and B, decapentaplegic(dpp), 60A, OP-2, dorsalin, growth differentiation factors (GDFs), nodal, MIS, inhibin-alpha, TGF-beta1, TGF-beta2, TGF-beta3, TGF-beta5, and glial-derived neurotrophic factor (GDNF).

Other preferred fragments are biologically active follistatin-3 fragments. Biologically active fragments are those exhibiting activity similar, but not necessarily

identical, to an activity of the follistatin-3 polypeptide. The biological activity of the fragments may include an improved desired activity, or a decreased undesirable activity.

Additionally, this invention provides a method of screening compounds to identify those which modulate the action of the polypeptide of the present invention. An example of such an assay comprises combining a mammalian fibroblast cell, a the polypeptide of the present invention, the compound to be screened and ^{3}[H] thymidine under cell culture conditions where the fibroblast cell would normally proliferate. A control assay may be performed in the absence of the compound to be screened and compared to the amount of fibroblast proliferation in the presence of the compound to determine if the compound stimulates proliferation by determining the uptake of [^{3}H] thymidine in each case. The amount of fibroblast cell proliferation is measured by liquid scintillation chromatography which measures the incorporation of [^{3}H] thymidine. Both agonist and antagonist compounds may be identified by this procedure.

In another method, a mammalian cell or membrane preparation expressing a receptor for a polypeptide of the present invention is incubated with a labeled polypeptide of the present invention in the presence of the compound. The ability of the compound to enhance or block this interaction could then be measured. Alternatively, the response of a known second messenger system following interaction of a compound to be screened and the follistatin-3 receptor is measured and the ability of the compound to bind to the receptor and elicit a second messenger response is measured to determine if the compound is a potential agonist or antagonist. Such second messenger systems include but are not limited to, cAMP guanylate cyclase, ion channels or phosphoinositide hydrolysis.

All of these above assays can be used as diagnostic or prognostic markers. The molecules discovered using these assays can be used to treat disease or to bring about a particular result in a patient (e.g., blood vessel growth) by activating or inhibiting the follistatin-3 /molecule. Moreover, the assays can discover agents which may inhibit or enhance the production of follistatin-3 from suitably manipulated cells or tissues.

Therefore, the invention includes a method of identifying compounds which bind to follistatin-3 comprising the steps of: (a) incubating a candidate binding compound with follistatin-3; and (b) determining if binding has occurred. Moreover, the invention

includes a method of identifying agonists/antagonists comprising the steps of: (a) incubating a candidate compound with follistatin-3, (b) assaying a biological activity , and (b) determining if a biological activity of follistatin-3 has been altered.

Antisense And Ribozyme (Antagonists).

In specific embodiments, antagonists according to the present invention are nucleic acids corresponding to the sequences contained in SEQ ID NO:1, or the complementary strand thereof, and/or to nucleotide sequences contained in the deposited clone follistatin-3. In one embodiment, antisense sequence is generated internally by the organism, in another embodiment, the antisense sequence is separately administered (see, for example, O'Connor, J., Neurochem. 56:560 (1991). Oligodeoxynucleotides as Anitsense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988). Antisense technology can be used to control gene expression through antisense DNA or RNA, or through triple-helix formation. Antisense techniques are discussed for example, in Okano, J., Neurochem. 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988). Triple helix formation is discussed in, for instance, Lee et al., Nucleic Acids Research 6:3073 (1979); Cooney et al., Science 241:456 (1988); and Dervan et al., Science 251:1300 (1991). The methods are based on binding of a polynucleotide to a complementary DNA or RNA.

For example, the 5' coding portion of a polynucleotide that encodes the mature polypeptide of the present invention may be used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription thereby preventing transcription and the production of the receptor. The antisense RNA oligonucleotide hybridizes to the mRNA in vivo and blocks translation of the mRNA molecule into receptor polypeptide.

In one embodiment, the follistatin-3 antisense nucleic acid of the invention is produced intracellularly by transcription from an exogenous sequence. For example, a vector or a portion thereof, is transcribed, producing an antisense nucleic acid (RNA) of the invention. Such a vector would contain a sequence encoding the follistatin-3 antisense nucleic acid. Such a vector can remain episomal or become chromosomally

integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others know in the art, used for replication and expression in vertebrate cells. Expression of the sequence encoding follistatin-3, or fragments thereof, can be by any promoter known in the art to act in vertebrate, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, Nature 29:304-310 (1981), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., Cell 22:787-797 (1980), the herpes thymidine promoter (Wagner et al., Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445 (1981), the regulatory sequences of the metallothionein gene (Brinster, et al., Nature 296:39-42 (1982)), etc.

The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of a follistatin-3 gene. However, absolute complementarity, although preferred, is not required. A sequence "complementary to at least a portion of an RNA," referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double stranded follistatin-3 antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the larger the hybridizing nucleic acid, the more base mismatches with a follistatin-3 RNA it may contain and still form a stable duplex (or triplex as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

Oligonucleotides that are complementary to the 5' end of the message, e.g., the 5' untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have been shown to be effective at inhibiting translation of mRNAs as well. See generally, Wagner, R., 1994, Nature 372:333-335. Thus, oligonucleotides complementary to either the 5'- or 3'- non- translated, non-coding regions of follistatin-3 shown in Figures 1A, 1B, and 1C could be used in an antisense

approach to inhibit translation of endogenous follistatin-3 mRNA. Oligonucleotides complementary to the 5' untranslated region of the mRNA should include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could be used in accordance with the invention. Whether designed to hybridize to the 5'-, 3'- or coding region of follistatin-3 mRNA, antisense nucleic acids should be at least six nucleotides in length, and are preferably oligonucleotides ranging from 6 to about 50 nucleotides in length. In specific aspects the oligonucleotide is at least 10 nucleotides, at least 17 nucleotides, at least 25 nucleotides or at least 50 nucleotides.

The polynucleotides of the invention can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide may include other appended groups such as peptides (e.g., for targeting host cell receptors in vivo), or agents facilitating transport across the cell membrane (see, e.g., Letsinger et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:6553-6556; Lemaitre et al., 1987, Proc. Natl. Acad. Sci. 84:648-652; PCT Publication No. WO88/09810, published December 15, 1988) or the blood-brain barrier (see, e.g., PCT Publication No. WO89/10134, published April 25, 1988), hybridization-triggered cleavage agents. (See, e.g., Krol et al., 1988, BioTechniques 6:958-976) or intercalating agents. (See, e.g., Zon, 1988, Pharm. Res. 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including, but not limited to, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-

D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including, but not limited to, arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the antisense oligonucleotide comprises at least one modified phosphate backbone selected from the group including, but not limited to, a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an a-anomeric oligonucleotide. An a-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual b-units, the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids Res. 15:6625-6641). The oligonucleotide is a 2'-0-methylribonucleotide (Inoue et al., 1987, Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al., 1987, FEBS Lett. 215:327-330).

Polynucleotides of the invention may be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein et al. (1988, Nucl. Acids Res. 16:3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc.

While antisense nucleotides complementary to the follistatin-3 coding region sequence could be used, those complementary to the transcribed untranslated region are most preferred.

Potential antagonists according to the invention also include catalytic RNA, or a ribozyme (See, e.g., PCT International Publication WO 90/11364, published October 4, 1990; Sarver et al, Science 247:1222-1225 (1990). While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy follistatin-3 mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Haseloff and Gerlach, Nature 334:585-591 (1988). There are numerous potential hammerhead ribozyme cleavage sites within the nucleotide sequence of follistatin-3 (Figures 1A, 1B, and 1C). Preferably, the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the follistatin-3 mRNA; i.e., to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

As in the antisense approach, the ribozymes of the invention can be composed of modified oligonucleotides (e.g. for improved stability, targeting, etc.) and should be delivered to cells which express follistatin-3 in vivo. DNA constructs encoding the ribozyme may be introduced into the cell in the same manner as described above for the introduction of antisense encoding DNA. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive promoter, such as, for example, pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous follistatin-3 messages and inhibit translation. Since ribozymes unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

Antagonist/agonist compounds may be employed to inhibit the cell growth and proliferation effects of the polypeptides of the present invention on neoplastic cells and tissues, i.e. stimulation of angiogenesis of tumors, and, therefore, retard or prevent abnormal cellular growth and proliferation, for example, in tumor formation or growth.

The antagonist/agonist may also be employed to prevent hyper-vascular diseases, and prevent the proliferation of epithelial lens cells after extracapsular cataract surgery.

Prevention of the mitogenic activity of the polypeptides of the present invention may also be desirous in cases such as restenosis after balloon angioplasty.

The antagonist/agonist may also be employed to prevent the growth of scar tissue during wound healing.

The antagonist/agonist may also be employed to treat the diseases described herein.

Other Activities.

The polypeptide of the present invention, as a result of the ability to stimulate vascular endothelial cell growth, may be employed in treatment for stimulating re-vascularization of ischemic tissues due to various disease conditions such as thrombosis, arteriosclerosis, and other cardiovascular conditions. These polypeptide may also be employed to stimulate angiogenesis and limb regeneration, as discussed above.

The polypeptide may also be employed for treating wounds due to injuries, burns, post-operative tissue repair, and ulcers since they are mitogenic to various cells of different origins, such as fibroblast cells and skeletal muscle cells, and therefore, facilitate the repair or replacement of damaged or diseased tissue.

The polypeptide of the present invention may also be employed stimulate neuronal growth and to treat and prevent neuronal damage which occurs in certain neuronal disorders or neuro-degenerative conditions such as Alzheimer's disease, Parkinson's disease, and AIDS-related complex. Follistatin-3 may have the ability to stimulate chondrocyte growth, therefore, they may be employed to enhance bone and periodontal regeneration and aid in tissue transplants or bone grafts.

The polypeptide of the present invention may be also be employed to prevent skin aging due to sunburn by stimulating keratinocyte growth.

The follistatin-3 polypeptide may also be employed for preventing hair loss, since FGF family members activate hair-forming cells and promotes melanocyte growth. Along the same lines, the polypeptides of the present invention may be employed to stimulate growth and differentiation of hematopoietic cells and bone marrow cells when used in combination with other cytokines.

The follistatin-3 polypeptide may also be employed to maintain organs before transplantation or for supporting cell culture of primary tissues.

The polypeptide of the present invention may also be employed for inducing tissue of mesodermal origin to differentiate in early embryos.

Follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, may also increase or decrease the differentiation or proliferation of embryonic stem cells, besides, as discussed above, hematopoietic lineage.

Follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, may also be used to modulate mammalian characteristics, such as body height, weight, hair color, eye color, skin, percentage of adipose tissue, pigmentation, size, and shape (e.g., cosmetic surgery). Similarly, follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, may be used to modulate mammalian metabolism affecting catabolism, anabolism, processing, utilization, and storage of energy.

Follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, may be used to change a mammal's mental state or physical state by influencing biorhythms, caricadic rhythms, depression (including depressive disorders), tendency for violence, tolerance for pain, reproductive capabilities (preferably by Activin or Inhibin-like activity), hormonal or endocrine levels, appetite, libido, memory, stress, or other cognitive qualities.

Follistatin-3 polynucleotides or polypeptides, or agonists or antagonists of follistatin-3, may also be used as a food additive or preservative, such as to increase or decrease storage capabilities, fat content, lipid, protein, carbohydrate, vitamins, minerals, cofactors or other nutritional components.

The above-recited applications have uses in a wide variety of hosts. Such hosts include, but are not limited to, human, murine, rabbit, goat, guinea pig, camel, horse, mouse, rat, hamster, pig, micro-pig, chicken, goat, cow, sheep, dog, cat, non-human primate, and human. In specific embodiments, the host is a mouse, rabbit, goat, guinea pig, chicken, rat, hamster, pig, sheep, dog or cat. In preferred embodiments, the host is a mammal. In most preferred embodiments, the host is a human.

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

Examples

Example 1(a): Expression and Purification of "His-tagged" Follistatin-3 in E. coli.

The bacterial expression vector pHE-4 is used for bacterial expression in this example. pHE-4 encodes ampicillin antibiotic resistance ("Amp^r") and contains a bacterial origin of replication ("ori"), an IPTG inducible promoter, a ribosome binding site ("RBS"), six codons encoding histidine residues that allow affinity purification using nickel-nitrilo-tri-acetic acid ("Ni-NTA") affinity resin sold by QIAGEN, Inc., *supra*, and suitable single restriction enzyme cleavage sites. These elements are arranged such that an inserted DNA fragment encoding a polypeptide expresses that polypeptide with the six His residues (i.e., a "6 X His tag") covalently linked to the amino terminus of that polypeptide.

The DNA sequence encoding the desired portion of the follistatin-3 protein comprising the mature form of the follistatin-3 amino acid sequence is amplified from the deposited cDNA clone using PCR oligonucleotide primers which anneal to the amino terminal sequences of the desired portion of the follistatin-3 protein and to sequences in the deposited construct 3' to the cDNA coding sequence. Additional nucleotides containing restriction sites to facilitate cloning in the pHE-4 vector are added to the 5' and 3' primer sequences, respectively.

For cloning the mature form of the follistatin-3 protein, the 5' primer has the sequence 5' TCA CGC CAT ATG GGC TCG GGG AAC C 3' (SEQ ID NO:12) containing the underlined *Nde* I restriction site followed by 16 nucleotides of the amino terminal coding sequence of the mature follistatin-3 sequence in SEQ ID NO:2. One of ordinary skill in the art would appreciate, of course, that the point in the protein coding sequence where the 5' primer begins may be varied to amplify a DNA segment encoding any desired portion of the complete follistatin-3 protein shorter or longer than the mature form of the protein. The 3' primer has the sequence 5' CAT CCG GGT ACC TTA TTA CAC GAA GTT CTC TTC CTC TTC TG 3' (SEQ ID NO:13) containing the underlined *Asp* 718 restriction site followed by two stop codons and 23 nucleotides complementary to the 3' end of the coding sequence of the follistatin-3 DNA sequence in Figure 1A.

The amplified follistatin-3 DNA fragment and the vector pHE4 are digested with *Nde* I and *Asp* 718 and the digested DNAs are then ligated together. Insertion of the follistatin-3 DNA into the restricted pHE4 vector places the follistatin-3 protein coding region downstream from the IPTG-inducible promoter and in-frame with an initiating AUG and the six histidine codons.

The ligation mixture is transformed into competent *E. coli* cells using standard procedures such as those described by Sambrook and colleagues (*Molecular Cloning: a Laboratory Manual, 2nd Ed.*; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989)). *E. coli* strain M15/rep4, containing multiple copies of the plasmid pREP4, which expresses the lac repressor and confers kanamycin resistance ("Kan^r"), is used in carrying out the illustrative example described herein. This strain, which is only one of many that are suitable for expressing follistatin-3 protein, is available commercially (QIAGEN, Inc., *supra*). Transformants are identified by their ability to grow on LB plates in the presence of ampicillin and kanamycin. Plasmid DNA is isolated from resistant colonies and the identity of the cloned DNA confirmed by restriction analysis, PCR and DNA sequencing.

Clones containing the desired constructs are grown overnight ("O/N") in liquid culture in LB media supplemented with both ampicillin (100 µg/ml) and kanamycin (25

μg/ml). The O/N culture is used to inoculate a large culture, at a dilution of approximately 1:25 to 1:250. The cells are grown to an optical density at 600 nm ("OD600") of between 0.4 and 0.6. Isopropyl-beta-D-thiogalactopyranoside ("IPTG") is then added to a final concentration of 1 mM to induce transcription from the lac repressor sensitive promoter, by inactivating the lacI repressor. Cells subsequently are incubated further for 3 to 4 hours. Cells then are harvested by centrifugation.

The cells are then stirred for 3-4 hours at 4°C in 6M guanidine-HCl, pH 8. The cell debris is removed by centrifugation, and the supernatant containing the follistatin-3 is loaded onto a nickel-nitrilo-tri-acetic acid ("Ni-NTA") affinity resin column (QIAGEN, Inc., *supra*). Proteins with a 6 x His tag bind to the Ni-NTA resin with high affinity and can be purified in a simple one-step procedure (for details see: The QIAexpressionist, 1995, QIAGEN, Inc., *supra*). Briefly the supernatant is loaded onto the column in 6 M guanidine-HCl, pH 8, the column is first washed with 10 volumes of 6 M guanidine-HCl, pH 8, then washed with 10 volumes of 6 M guanidine-HCl pH 6, and finally the follistatin-3 is eluted with 6 M guanidine-HCl, pH 5.

The purified protein is then renatured by dialyzing it against phosphate-buffered saline (PBS) or 50 mM Na-acetate, pH 6 buffer plus 200 mM NaCl. Alternatively, the protein can be successfully refolded while immobilized on the Ni-NTA column. The recommended conditions are as follows: renature using a linear 6M-1M urea gradient in 500 mM NaCl, 20% glycerol, 20 mM Tris/HCl pH 7.4, containing protease inhibitors. The renaturation should be performed over a period of 1.5 hours or more. After renaturation the proteins can be eluted by the addition of 250 mM immidazole. Immidazole is removed by a final dialyzing step against PBS or 50 mM sodium acetate pH 6 buffer plus 200 mM NaCl. The purified protein is stored at 4° C or frozen at -80° C.

The following alternative method may be used to purify follistatin-3 expressed in *E coli* when it is present in the form of inclusion bodies. Unless otherwise specified, all of the following steps are conducted at 4-10°C.

Upon completion of the production phase of the *E. coli* fermentation, the cell culture is cooled to 4-10°C and the cells are harvested by continuous centrifugation at 15,000 rpm (Heraeus Sepatech). On the basis of the expected yield of protein per unit weight of cell paste and the amount of purified protein required, an appropriate amount of cell paste, by weight, is suspended in a buffer solution containing 100 mM Tris, 50 mM EDTA, pH 7.4. The cells are dispersed to a homogeneous suspension using a high shear mixer.

The cells ware then lysed by passing the solution through a microfluidizer (Microfluidics, Corp. or APV Gaulin, Inc.) twice at 4000-6000 psi. The homogenate is then mixed with NaCl solution to a final concentration of 0.5 M NaCl, followed by centrifugation at 7000 x *g* for 15 min. The resultant pellet is washed again using 0.5M NaCl, 100 mM Tris, 50 mM EDTA, pH 7.4.

The resulting washed inclusion bodies are solubilized with 1.5 M guanidine hydrochloride (GuHCl) for 2-4 hours. After 7000 x *g* centrifugation for 15 min., the pellet is discarded and the follistatin-3 polypeptide-containing supernatant is incubated at 4°C overnight to allow further GuHCl extraction.

Following high speed centrifugation (30,000 x *g*) to remove insoluble particles, the GuHCl solubilized protein is refolded by quickly mixing the GuHCl extract with 20 volumes of buffer containing 50 mM sodium, pH 4.5, 150 mM NaCl, 2 mM EDTA by vigorous stirring. The refolded diluted protein solution is kept at 4°C without mixing for 12 hours prior to further purification steps.

To clarify the refolded follistatin-3 polypeptide solution, a previously prepared tangential filtration unit equipped with 0.16 micrometer membrane filter with appropriate surface area (e.g., Filtron), equilibrated with 40 mM sodium acetate, pH 6.0 is employed. The filtered sample is loaded onto a cation exchange resin (e.g., Poros HS-50, Perseptive Biosystems). The column is washed with 40 mM sodium acetate, pH 6.0 and eluted with 250 mM, 500 mM, 1000 mM, and 1500 mM NaCl in the same buffer, in a stepwise manner. The absorbance at 280 nm of the effluent is continuously monitored. Fractions are collected and further analyzed by SDS-PAGE.

Fractions containing the follistatin-3 polypeptide are then pooled and mixed with 4 volumes of water. The diluted sample is then loaded onto a previously prepared set of tandem columns of strong anion (Poros HQ-50, Perseptive Biosystems) and weak anion (Poros CM-20, Perseptive Biosystems) exchange resins. The columns are equilibrated with 40 mM sodium acetate, pH 6.0. Both columns are washed with 40 mM sodium acetate, pH 6.0, 200 mM NaCl. The CM-20 column is then eluted using a 10 column volume linear gradient ranging from 0.2 M NaCl, 50 mM sodium acetate, pH 6.0 to 1.0 M NaCl, 50 mM sodium acetate, pH 6.5. Fractions are collected under constant A_{280} monitoring of the effluent. Fractions containing the follistatin-3 polypeptide (determined, for instance, by 16% SDS-PAGE) are then pooled.

The resultant follistatin-3 polypeptide exhibits greater than 95% purity after the above refolding and purification steps. No major contaminant bands are observed from Commassie blue stained 16% SDS-PAGE gel when 5 micrograms of purified protein is loaded. The purified protein is also tested for endotoxin/LPS contamination, and typically the LPS content is less than 0.1 ng/ml according to LAL assays.

Example 2: Cloning and Expression of Follistatin-3 protein in a Baculovirus Expression System.

In this illustrative example, the plasmid shuttle vector pA2 is used to insert the cloned DNA encoding complete protein, including its naturally associated secretory signal (leader) sequence, into a baculovirus to express the mature follistatin-3 protein, using standard methods as described by Summers and colleagues (*A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, Texas Agricultural Experimental Station Bulletin No. 1555 (1987)). This expression vector contains the strong polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) followed by convenient restriction sites such as *Bam* HI, *Xba* I and *Asp* 718. The polyadenylation site of the simian virus 40 ("SV40") is used for efficient polyadenylation. For easy selection of recombinant virus, the plasmid contains the beta-galactosidase gene from *E. coli* under control of a weak Drosophila promoter in the

same orientation, followed by the polyadenylation signal of the polyhedrin gene. The inserted genes are flanked on both sides by viral sequences for cell-mediated homologous recombination with wild-type viral DNA to generate a viable virus that express the cloned polynucleotide.

Many other baculovirus vectors could be used in place of the vector above, such as pAc373, pVL941 and pAcIM1, as one skilled in the art would readily appreciate, as long as the construct provides appropriately located signals for transcription, translation, secretion and the like, including a signal peptide and an in-frame AUG as required. Such vectors are described, for instance, by Luckow and coworkers (*Virology* **170**:31-39 (1989)).

The cDNA sequence encoding the full length follistatin-3 protein in the deposited clone, including the AUG initiation codon and the naturally associated leader sequence shown in SEQ ID NO:2, is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene. The 5' primer has the sequence 5' CAT CGC GGA TCC GCC ATC ATG CGT CCC GGG GCG CCA GGG C 3' (SEQ ID NO:14) containing the underlined *Bam* HI restriction enzyme site, an efficient signal for initiation of translation in euksryotic cells (Kozak, M., *J. Mol. Biol.* **196**:947-950 (1987)), followed by 22 of nucleotides of the sequence of the complete follistatin-3 protein shown in Figure 1A, beginning with the AUG initiation codon. The 3' primer has the sequence 5' CAT CCG GGT ACC TCA CAC GAA GTT CTC TTC CTC TTC TG 3' (SEQ ID NO:15) containing the underlined *Asp* 718 restriction site followed by 23 nucleotides complementary to the 3' noncoding sequence in Figure 1A.

The amplified fragment is isolated from a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101 Inc., La Jolla, Ca.). The fragment then is digested with *Bam* HI and *Asp* 718 and again is purified on a 1% agarose gel. This fragment is designated herein F1.

The plasmid is digested with the restriction enzymes *Bam* HI and *Asp* 718 and optionally, can be dephosphorylated using calf intestinal phosphatase, using routine procedures known in the art. The DNA is then isolated from a 1% agarose gel using a

commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.). This vector DNA is designated herein "V1".

Fragment F1 and the dephosphorylated plasmid V1 are ligated together with T4 DNA ligase. *E. coli* HB101 or other suitable *E. coli* hosts such as XL-1 Blue (Statagene Cloning Systems, La Jolla, CA) cells are transformed with the ligation mixture and spread on culture plates. Bacteria are identified that contain the plasmid with the human follistatin-3 gene by digesting DNA from individual colonies using *Bam* HI and *Asp* 718 and then analyzing the digestion product by gel electrophoresis. The sequence of the cloned fragment is confirmed by DNA sequencing. This plasmid is designated herein pA2Follistatin-3.

Five µg of the plasmid pA2Follistatin-3 is co-transfected with 1.0 µg of a commercially available linearized baculovirus DNA ("BaculoGold™ baculovirus DNA", Pharmingen, San Diego, CA), using the lipofection method described by Felgner and colleaguew (*Proc. Natl. Acad. Sci. USA* **84**:7413-7417 (1987)). One µg of BaculoGold™ virus DNA and 5 µg of the plasmid pA2Follistatin-3 are mixed in a sterile well of a microtiter plate containing 50 µl of serum-free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards, 10 µl Lipofectin plus 90 µl Grace's medium are added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture is added drop-wise to Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate is then incubated for 5 hours at 27°C. The transfection solution is then removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum is added. Cultivation is then continued at 27°C for four days.

After four days the supernatant is collected and a plaque assay is performed, as described by Summers and Smith (*supra*). An agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) is used to allow easy identification and isolation of gal-expressing clones, which produce blue-stained plaques. (A detailed description of a "plaque assay" of this type can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10). After

appropriate incubation, blue stained plaques are picked with the tip of a micropipettor (e.g., Eppendorf). The agar containing the recombinant viruses is then resuspended in a microcentrifuge tube containing 200 µl of Grace's medium and the suspension containing the recombinant baculovirus is used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes are harvested and then they are stored at 4°C. The recombinant virus is called V-Follistatin-3.

To verify the expression of the follistatin-3 gene Sf9 cells are grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells are infected with the recombinant baculovirus V-Follistatin-3 at a multiplicity of infection ("MOI") of about 2. If radiolabeled proteins are desired, 6 hours later the medium is removed and is replaced with SF900 II medium minus methionine and cysteine (available from Life Technologies Inc., Rockville, MD). After 42 hours, 5 µCi of ^{35}S-methionine and 5 µCi ^{35}S-cysteine (available from Amersham) are added. The cells are further incubated for 16 hours and then are harvested by centrifugation. The proteins in the supernatant as well as the intracellular proteins are analyzed by SDS-PAGE followed by autoradiography (if radiolabeled).

Microsequencing of the amino acid sequence of the amino terminus of purified protein may be used to determine the amino terminal sequence of the mature form of the follistatin-3 protein, and thus the cleavage point and length of the naturally associated secretory signal peptide.

Example 3: Cloning and Expression of Follistatin-3 in Mammalian Cells.

A typical mammalian expression vector contains the promoter element, which mediates the initiation of transcription of mRNA, the protein coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription can be achieved with the early and late promoters from SV40, the long terminal repeats (LTRs) from Retroviruses, e.g., RSV, HTLVI, HIVI and the early promoter of the

cytomegalovirus (CMV). However, cellular elements can also be used (e.g., the human actin promoter). Suitable expression vectors for use in practicing the present invention include, for example, vectors such as pSVL and pMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146) and pBC12MI (ATCC 67109). Mammalian host cells that could be used include, human Hela, 293, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7 and CV1, quail QC1-3 cells, mouse L cells and Chinese hamster ovary (CHO) cells.

Alternatively, the gene can be expressed in stable cell lines that contain the gene integrated into a chromosome. The co-transfection with a selectable marker such as dhfr, gpt, neomycin, hygromycin allows the identification and isolation of the transfected cells.

The transfected gene can also be amplified to express large amounts of the encoded protein. The DHFR (dihydrofolate reductase) marker is useful to develop cell lines that carry several hundred or even several thousand copies of the gene of interest. Another useful selection marker is the enzyme glutamine synthase (GS; Murphy, *et al.*, *Biochem J.* **227**:277-279 (1991); Bebbington, *et al.*, *Bio/Technology* **10**:169-175 (1992)). Using these markers, the mammalian cells are grown in selective medium and the cells with the highest resistance are selected. These cell lines contain the amplified gene(s) integrated into a chromosome. Chinese hamster ovary (CHO) and NSO cells are often used for the production of proteins.

The expression vectors pC1 and pC4 contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen, *et al.*, *Mol. Cel. Biol.* **5**:438-447 (1985)) plus a fragment of the CMV-enhancer (Boshart, *et al.*, *Cell* **41**:521-530 (1985)). Multiple cloning sites, e.g., with the restriction enzyme cleavage sites *Bam* HI, *Xba* I and *Asp* 718, facilitate the cloning of the gene of interest. The vectors contain in addition the 3' intron, the polyadenylation and termination signal of the rat preproinsulin gene.

Example 3(a): Cloning and Expression in COS Cells

The expression plasmid, pFollistatin-3HA, is made by cloning a portion of the cDNA encoding the mature form of the follistatin-3 protein into the expression vector pcDNAI/Amp or pcDNAIII (which can be obtained from Invitrogen, Inc.).

The expression vector pcDNAI/amp contains: (1) an *E. coli* origin of replication effective for propagation in *E. coli* and other prokaryotic cells; (2) an ampicillin resistance gene for selection of plasmid-containing prokaryotic cells; (3) an SV40 origin of replication for propagation in eukaryotic cells; (4) a CMV promoter, a polylinker, an SV40 intron; (5) several codons encoding a hemagglutinin fragment (i.e., an "HA" tag to facilitate purification) followed by a termination codon and polyadenylation signal arranged so that a cDNA can be conveniently placed under expression control of the CMV promoter and operably linked to the SV40 intron and the polyadenylation signal by means of restriction sites in the polylinker. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein described by Wilson and colleagues (*Cell* **37**:767 (1984)). The fusion of the HA tag to the target protein allows easy detection and recovery of the recombinant protein with an antibody that recognizes the HA epitope. pcDNAIII contains, in addition, the selectable neomycin marker.

A DNA fragment encoding the complete follistatin-3 polypeptide is cloned into the polylinker region of the vector so that recombinant protein expression is directed by the CMV promoter. The plasmid construction strategy is as follows. The follistatin-3 cDNA of the deposited clone is amplified using primers that contain convenient restriction sites, much as described above for construction of vectors for expression of follistatin-3 in *E. coli*. Suitable primers include the following, which are used in this example. The 5' primer, containing the underlined *Bam* HI site, a Kozak sequence, an AUG start codon, and 22 nucleotides of the 5' coding region of the complete follistatin-3 polypeptide, has the following sequence: 5'-CAT CGC GGA TCC GCC ACC ATG CGT CCC GGG GCG CCA GGG C-3' (SEQ ID NO:16). The 3' primer, containing the underlined *Asp* 718 and 23 of nucleotides complementary to the 3' coding sequence immediately before the stop codon, has the following sequence: 5'-TCA CCG CTC GAG CAC GAA GTT CTC TTC CTC TTC TG-3' (SEQ ID NO:17).

The PCR amplified DNA fragment and the vector, pcDNAI/Amp, are digested with *Bam* HI and *Asp* 718 and then ligated. The ligation mixture is transformed into *E. coli* strain SURE (Stratagene Cloning Systems, La Jolla, CA 92037), and the transformed culture is plated on ampicillin media plates which then are incubated to allow growth of ampicillin resistant colonies. Plasmid DNA is isolated from resistant colonies and examined by restriction analysis or other means for the presence of the fragment encoding the complete follistatin-3 polypeptide

For expression of recombinant follistatin-3, COS cells are transfected with an expression vector, as described above, using DEAE-dextran, as described, for instance, by Sambrook and coworkers (*Molecular Cloning: a Laboratory Manual*, Cold Spring Laboratory Press, Cold Spring Harbor, New York (1989)). Cells are incubated under conditions for expression of follistatin-3 by the vector.

Expression of the follistatin-3-HA fusion protein is detected by radiolabeling and immunoprecipitation, using methods described in, for example Harlow and colleagues (*Antibodies: A Laboratory Manual, 2nd Ed.*; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1988)). To this end, two days after transfection, the cells are labeled by incubation in media containing ^{35}S-cysteine for 8 hours. The cells and the media are collected, and the cells are washed and the lysed with detergent-containing RIPA buffer: 150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50 mM TRIS, pH 7.5, as described by Wilson and colleagues (*supra*). Proteins are precipitated from the cell lysate and from the culture media using an HA-specific monoclonal antibody. The precipitated proteins then are analyzed by SDS-PAGE and autoradiography. An expression product of the expected size is seen in the cell lysate, which is not seen in negative controls.

Example 3(b): Cloning and Expression in CHO Cells

The vector pC4 is used for the expression of follistatin-3 polypeptide. Plasmid pC4 is a derivative of the plasmid pSV2-dhfr (ATCC Accession No. 37146). The plasmid contains the mouse DHFR gene under control of the SV40 early promoter. Chinese hamster ovary- or other cells lacking dihydrofolate activity that are transfected

with these plasmids can be selected by growing the cells in a selective medium (alpha minus MEM, Life Technologies) supplemented with the chemotherapeutic agent methotrexate. The amplification of the DHFR genes in cells resistant to methotrexate (MTX) has been well documented (see, e.g., Alt, F. W., *et al., J. Biol. Chem.* **253**:1357-1370 (1978); Hamlin, J. L. and Ma, C. *Biochem. et Biophys. Acta,* **1097**:107-143 (1990); Page, M. J. and Sydenham, M. A. *Biotechnology* **9**:64-68 (1991)). Cells grown in increasing concentrations of MTX develop resistance to the drug by overproducing the target enzyme, DHFR, as a result of amplification of the DHFR gene. If a second gene is linked to the DHFR gene, it is usually co-amplified and over-expressed. It is known in the art that this approach may be used to develop cell lines carrying more than 1,000 copies of the amplified gene(s). Subsequently, when the methotrexate is withdrawn, cell lines are obtained which contain the amplified gene integrated into one or more chromosome(s) of the host cell.

Plasmid pC4 contains for expressing the gene of interest the strong promoter of the long terminal repeat (LTR) of the Rouse Sarcoma Virus (Cullen, *et al., Mol. Cell. Biol.* **5**:438-447 (1985)) plus a fragment isolated from the enhancer of the immediate early gene of human cytomegalovirus (CMV; Boshart, *et al., Cell* **41**:521-530 (1985)). Downstream of the promoter are the following single restriction enzyme cleavage sites that allow the integration of the genes: *Bam* HI, *Xba* I, and *Asp* 718. Behind these cloning sites the plasmid contains the 3' intron and polyadenylation site of the rat preproinsulin gene. Other high efficiency promoters can also be used for the expression, e.g., the human ß-actin promoter, the SV40 early or late promoters or the long terminal repeats from other retroviruses, e.g., HIV and HTLVI. Clontech's Tet-Off and Tet-On gene expression systems and similar systems can be used to express the follistatin-3 polypeptide in a regulated way in mammalian cells (Gossen, M., and Bujard, H. *Proc. Natl. Acad. Sci. USA* **89**:5547-5551 (1992)). For the polyadenylation of the mRNA other signals, e.g., from the human growth hormone or globin genes can be used as well. Stable cell lines carrying a gene of interest integrated into the chromosomes can also be selected upon co-transfection with a selectable marker such as gpt, G418 or hygromycin.

It is advantageous to use more than one selectable marker in the beginning, e.g., G418 plus methotrexate.

The plasmid pC4 is digested with the restriction enzymes *Bam* HI and *Asp* 718 and then dephosphorylated using calf intestinal phosphates by procedures known in the art. The vector is then isolated from a 1% agarose gel.

The DNA sequence encoding the complete follistatin-3 polypeptide is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the desired portion of the gene. The 5' primer containing the underlined *Bam* HI site, a Kozak sequence, an AUG start codon, and 22 nucleotides of the 5' coding region of the complete follistatin-3 polypeptide, has the following sequence: 5' CAT CGC GGA TCC *GCC ACC* ATG CGT CCC GGG GCG CCA GGG C 3' (SEQ ID NO:18). The 3' primer, containing the underlined *Asp* 718 restriction site and 23 of nucleotides complementary to the 3' coding sequence immediately before the stop codon as shown in Figure 1A (SEQ ID NO:1), has the following sequence: 5' CAT CCG GGT ACC TCA CAC GAA GTT CTC TTC CTC TTC TG 3' (SEQ ID NO:19).

The amplified fragment is digested with the endonucleases *Bam* HI and *Asp* 718 and then purified again on a 1% agarose gel. The isolated fragment and the dephosphorylated vector are then ligated with T4 DNA ligase. *E. coli* HB101 or XL-1 Blue cells are then transformed and bacteria are identified that contain the fragment inserted into plasmid pC4 using, for instance, restriction enzyme analysis.

Chinese hamster ovary cells lacking an active DHFR gene are used for transfection. Five µg of the expression plasmid pC4 is cotransfected with 0.5 µg of the plasmid pSVneo using lipofectin (Felgner, *et al., supra*). The plasmid pSV2-neo contains a dominant selectable marker, the *neo* gene from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells are seeded in alpha minus MEM supplemented with 1 mg/ml G418. After 2 days, the cells are trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) in alpha minus MEM supplemented with 10, 25, or 50 ng/ml of metothrexate plus 1 mg/ml G418. After about 10-14 days single clones are trypsinized and then seeded in 6-well petri dishes or

10 ml flasks using different concentrations of methotrexate (50 nM, 100 nM, 200 nM, 400 nM, 800 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new 6-well plates containing even higher concentrations of methotrexate (1 μM, 2 μM, 5 μM, 10 mM, 20 mM). The same procedure is repeated until clones are obtained which grow at a concentration of 100-200 μM. Expression of the desired gene product is analyzed, for instance, by SDS-PAGE and Western blot or by reversed phase HPLC analysis.

Example 4: Tissue distribution of Follistatin-3 mRNA expression.

Northern blot analysis was carried out to examine follistatin-3 gene expression in human tissues, using methods described by, among others, Sambrook and colleagues (*supra*). A cDNA probe containing the entire nucleotide sequence of the follistatin-3 protein (SEQ ID NO:1) was labeled with ^{32}P using the *rediprime*™ DNA labeling system (Amersham Life Science), according to manufacturer's instructions. After labeling, the probe was purified using a CHROMA SPIN-100™ column (Clontech Laboratories, Inc.), according to manufacturer's protocol number PT1200-1. The purified labeled probe was then used to examine various human tissues for follistatin-3 mRNA.

Multiple Tissue Northern (MTN) blots containing various human tissues (H) or human immune system tissues (IM) were obtained from Clontech and were examined with the labeled probe using ExpressHyb™ hybridization solution (Clontech) according to manufacturer's protocol number PT1190-1. Following hybridization and washing, the blots were mounted and exposed to film at -70°C overnight, and films developed according to standard procedures. The follistatin-3-specific probe recognized an mRNA species of approximately 2.6 kb in most tissues examined.

Example 5: Follistatin-3 encodes an activin-binding protein that is differentially regulated compared to follistatin.

Follistatin is a glycosylated, single chain polypeptide that was discovered as an activin-binding protein in the rat ovary. (Vale, W., *et al.*, *Nature* 321, 776-79 (1986); Ling, N., *et al.*, *Nature* 321, 779-82 (1986); Ueno, N., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 84,

8282-86 (1987); Esch, F.S., *et al.*, *Mol. Endocrinol.* 1, 849-55 (1987); Nakamura, T., *et al.*, *Science* 247, 836-38 (1990), reviewed in Michel, V., *et al.*, *Molec. Cell. Endocrinol.* 91, 1-11 (1993)). It was shown to inhibit the release of follicle-stimulating hormone (FSH) from the pituitary by binding to activins (Nakamura, T., *et al.*, *Science* 247, 836-38 (1990); Kogawa, K., *et al.*, *Endocrinol.* 128, 1434-40 (1991), reviewed in Vale, W., *et al.*, *The inhibin/activin family of hormones and growth factors*, In Peptide Growth Factors and Their Receptors II, eds. Sporn, M.B. and Roberts, A.B., (Springer-Verlag) (1990)), indicating that follistatin inhibits activin actions. This has now been confirmed in *in vitro* and *in vivo* experiments (Nakamura, T., *et al.*, *Science* 247, 836-38 (1990); de Winter, J. P., *et al.*, *Mol. Cell. Endocrinol.* 116, 105-14 (1996); for review see Patel, K. *Int. J. Biochem. Cell Biol.* 30, 1087-93 (1998)). Follistatin prevents binding of activins to their high affinity transmembrane receptors (de Winter, J. P., *et al.*, *Mol. Cell. Endocrinol.* 116, 105-14 (1996)) and it also facilitates clearance of activins by accelerating their endocytotic degradation (Hashimoto, O., *et al.*, *J. Biol. Chem.* 272, 13835-13842 (1997)).

Two forms of follistatin have been identified, follistatin-315 (FS-315) and a carboxyterminally truncated variant, follistatin-288 (FS-288). These two follistatin molecules result from alternative splicing of one primary transcript (Shimasaki, S., *et al.*, *Proc., Natl., Acad. Sci. U.S.A.* 85, 4218-22 (1998)). Among several differences, FS-288 was shown to be more potent than FS-315 in suppressing FSH release in rat anterior pituitary cells (Sugino, K., *et al.*, *J. Biol. Chem.* 268, 15579-87 (1993)). In addition to its activin-binding activity, follistatin binds to other members of the transforming growth factor (TGF)-beta superfamily such as bone morphogenetic proteins 2, 4, and 7 (Fainsod, A., *et al.*, *Mech. Dev.* 63, 39-50 (1997)). The *in vivo* relevance of these interactions is as yet unclear. The physiological role of follistatin is not limited to the inhibition of FSH release. For example, overexpression of follistatin in the developing *Xenopus laevis* embryo led to induction of neural tissue (Hemmati-Brivanlou, A., *et al.*, *Cell* 77, 283-95 (1994)). Furthermore, follistatin-deficient mice showed multiple defects and perinatal death (Matzuk, M.M., *et al.*, *Nature* 374, 360-63 (1995)). The defects include those in the muscles, skin, bone and teeth. These data are consistant with the hypothesis that follistatin is involved in multiple physiological functions.

We have recently identified a human follistatin homologue, follistatin-3, from the Human Genome Sciences expressed sequence tag (EST) database (EST clone number HDTAH85). The cDNA of this follistatin homologue is 2.6 kb in length and encodes a protein of 263 amino acids. Hayette et al. have also reported the Follistatin-Related Gene (FLRG) (Hayette, S., *et al.*, *Oncogene* 16, 2949-54 (1998)) whose sequence was identical to our cDNA. FLRG was identified by positional cloning of transcriptional units on chromosome 19 at the t(11;19)(q13;p13) translocation breakpoint. This breakpoint was observed in a case of B cell chronic lymphocytic leukemia. Hayette et al. have also reported on the structural rearrangement of the FLRG locus in a case of non-Hodgkin's lymphoma. These data suggest that FLRG may play a role in leukemogenesis.

In this study, we demonstrate that FLRG is a functional activin-binding protein which, like follistatin, binds both activin A and activin B. However, we demonstrate differential expression in tissues and regulation of follistatin and FLRG expression in cultured keratinocytes. Our results indicate differences in the *in vivo* regulation and functions of FLRG and follistatin proteins.

Materials and Methods

Molecular Cloning of Follistatin-3. Searches of the Human Genome Sciences database of expressed sequence tags (ESTs) identified a cDNA clone from a Hodgkin's Lymphoma II library, HDTAH85, that was homologous to part of follistatin. We named this clone follistatin-3. A cDNA probe encompassing the first 417 nucleotides of the predicted coding sequence of follistatin-3 was isolated by performing two rounds of PCR from a human adult liver cDNA library. This FLRG-specific probe was used to obtain a longer clone containing the complete coding sequence and the 3' non-coding region of follistatin-3 from a fetal bone cDNA library.

Transfection and immunoprecipitation experiments. For transfection into mammalian cells, the FLRG open reading frame without 5'- or 3'-untranslated regions was cloned into pcDNA3 vector (Invitrogen) with an in-frame epitope tag , HA (YPYDVPDYA) (SEQ ID NO:20), or Flag (DYKDDDDK) (SEQ ID NO:21) at the carboxyl terminus. Likewise, the human activin betaA open reading frame without 5'- or 3'-untranslated regions was cloned into pcDNA3 vector (Invitrogen) with an in-frame HA tag , and the human activin betaB

with an in-frame HA tag or a myc tag (EQKLISEEDL) (SEQ ID NO:22), at the carboxyl termini. Human kidney epithelial 293 cells (ATCC), grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum (FCS), were transfected with expression constructs, such as pcDNA-FLRG-HA, pcDNA-FLRG-Flag, or combinations of FLRG constructs with activin betaA or activin betaB constructs. The transfection was performed using the Lipofectamine method (Life Technology) according to the manufacturer's instructions. The cell culture media were collected 48 hours post-transfection. Cells were harvested at the same time as the culture media. Transfected c ells were lysed in NP-40 lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 1% NP-40, 10% Glycerol, 1 mM PMSF, Leupeptin 1.0 µg/ml, EDTA 0.5mM, NaF 1 mM, E64 1 µg/ml, Aprorinin 1µg/ml sodium orthovanadate 1mM,). FLRG proteins in the cell culture supernatants and cell lysates were immunoprecipitated with a monoclonal anti-HA antibody (Roche, Mannheim, Germany; 2 micrograms/10 ml for cell supernatants and 1 microgram/ml for cell lysates), or a monoclonal anti-Flag antibody (Kodak-IBI). The immunoprecipitates were separated by reducing SDS PAGE and subsequently by Western blot analyses.

Anti-FLRG antibody. Purified FLRG protein from Baculoviraus/insect cell expression system was used to immunize rabbits. The anti-serum was used at 1: 7,500 dilution for Western blotting.

Northern blot analysis. Three Clontech human multiple tissue Northern blots were hybridized with ^{32}P-labelled FLRG and follistatin cDNA probes as described by the manufacturer.

Cell culture for RNase protection assays. The immortalized but non-transformed human HaCaT keratinocyte cell line (18) was used for all tissue culture experiments. Cells were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum. For the analysis of follistatin and FLRG regulation, cells were grown to confluency in 10-cm culture dishes and rendered quiescent by a 16 hr incubation in serum-free DMEM. Cells were subsequently incubated for varying periods in fresh DMEM containing 20 ng/ml epidermal growth factor (EGF), 10 ng/ml keratinocyte growth factor

(KGF), or 1 ng/ml transforming growth factor beta1 (TGF-beta1). Aliquots of cells were harvested before and at different time points after treatment with these reagents and used for RNA isolation. FCS and DMEM were purchased from Life Technologies, Inc. Growth factors and cytokines were from Roche (Mannheim, Germany). Each experiment was repeated at least twice.

RNA isolation and RNase protection assay. Total cellular RNA was isolated from HaCaT cells as described by Chomczynski and Sacchi (Chomczynski, P. and Sacchi, N. *Anal. Biochem.* 162, 156-59 (1987)). RNase protection assays were carried out as described (Werner, S., *et al.*, *EMBO J.* 12, 2635-43 (1993)). All protection assays were repeated with a different set of RNAs from independent experiments. A 247 bp fragment corresponding to nt 28-274 of the human FLRG cDNA and a 253 bp fragment corresponding to nt 28-280 of the follistatin gene (Shimasaki, S., *et al.*, *Proc., Natl., Acad. Sci. U.S.A.* 85, 4218-22 (1998)) were used as probes.

Results and Discussion

Expression of FLRG in mammalian cells

To elucidate the functions of FLRG, we first expressed FLRG protein in mammalian cells. An expression vector containing the entire open reading frame of FLRG fused to a C-terminal epitope tag, Flag, was transfected into 293 human kidney epithelial cell. Cell lysates and cell culture media were analyzed by Western blot analyses. In both cell lysates and cell culture media, FLRG appeared as a diffuse band of approximately 35 kDa on a Western blot probed with an anti-FLRG antibody. The same 35 kDa band was detected with an anti-tag antibody that recognized the C-terminal Flag-tag on FLRG. The amino acid sequence of FLRG contains a signal peptide and therefore predicts that FLRG is a secreted protein. Our results showed that the FLRG protein was indeed secreted into the cell culture media. However, unlike the intracellular FLRG protein, the secreted FLRG detected by the anti-Flag antibody showed a much weaker signal than that detected by the polyclonal anti-FLRG antibody. One hypothesis for this observation could be that part of the secreted FLRG may have a proteolytically clipped C-terminus. The predicted molecular weight of FLRG is 27.6 kDa. The difference between the predicted and the apparent molecular weight (35 kDa)

could be accounted for by glycosylation of the FLRG protein. In fact, the FLRG amino acid sequence contains two predicted N-glycosylation sites (aa 73 and aa 215) (Hayette, S., *et al.*, *Oncogene* 16, 2949-54 (1998)). Furthermore, thue diffuseness of the FLRG protein band on the Western blot also suggests that FLRG is glycosylated.

Binding of FLRG to activin A and B

Activin exists as homo- or heterodimers of a betaA and a betaB chain. Three activins, activin A (betaAbetaA homodimer), activin B (betaBbetaB homodimer), and activin AB (betaAbetaB heterodimer) all bind follistatin with high affinities. Activins are synthesized as large precursor polypeptides which are proteolytically processed into mature polypeptides. The disulfide-linked dimers of mature activins form the biologically active molecules of approximately 28 kDa. Under reducing conditions, the dimers can be separated into two subunits of approximately 14 kDa (reviewed in Vale, W., *et al.*, *The inhibin/activin family of hormones and growth factors*, In Peptide Growth Factors and Their Receptors II, eds. Sporn, M.B. and Roberts, A.B., (Springer-Verlag) (1990)).

To determine whether FLRG is able to bind activins, 293 cells were co-transfected with a C-terminal Flag-tagged FLRG construct with either an activin betaA-HA or with an activin betaB-myc expression construct. Flag-tagged follistatin-315 (FS-315) and Flag-tagged follistatin-288 (FS-288), were used as positive controls. These molecules were immunoprecipitated from either the cell culture media or cell lysates with an anti-Flag monoclonal antibody. The co-immunoprecipitated activins A or B were then detected on a Western blot by anti-tag antibodies. Both positive controls, FS-315 and FS-288, co-immunoprecipitated the mature form of Flag-tagged activin A which appeared on the Western blot as the 16 kDa monomer under reducing conditions. In addition, we made a novel observation that FS-315 and FS-288 immunoprecipitated a protein of approximately 55 kDa from the cell lysates. The latter most likely represents the reduced form of activin A precursor. Although this precursor was reported to be inactive in releasing follicle-stimulating hormone from pituitary cells in *in vitro* assays (Mason, A. J., *et al.*, *Mol. Endocrinol.* 10: 1055-65 (1996)), follistatin binding to the precursor may prevent the processing of the precursor into the active mature form of activin A. This may, in turn, further inhibit activin A activity.

In cell lysates where FLRG was co-transfected with the activin-betaA cDNA, FLRG co-immunoprecipitated activin A. The ability of FLRG to associate with activin A was comparable to FS-315 and FS-288, as judged from the amounts of FLRG, FS-315, and FS-288 proteins in the immunoprecipitates. The major activin A species precipitated from cell lysates by FLRG was the high molecular weight form of ~55 kDa under reducing conditions. In control experiments where activin betaA was transfected alone or co-transfected with pcDNA vector, no activin A was immunoprecipitated by the anti-Flag antibody that recognized FLRG. These results demonstrate that FLRG, like FS-315 and FS-288, can bind the unprocessed high molecular weight activin A precursor.

In the cell culture media, secreted FLRG, as well as FS-315 and FS-288, co-immunoprecipitated the secreted low molecular weight mature form activin A, as demonstrated by the detection of the 16 kDa monomeric Flag-tagged activin betaA protein on the Western blot. In addition to the 16 kDa species, FS-288 also precipitated a secreted Flag-tagged activin betaA species of approximately 20 kDa.

In cell lysates where FLRG was co-transfected with activin betaB, FLRG co-immunoprecipitated activin B precursor molecules of 48 – 55 kDa. Judging from the amounts of FLRG, FS-315, and FS-288 proteins in the immunoprecipitates, the ability of FLRG to co-immunoprecipitate activin B was at least as good as that of FS-315 and possibly even better than that of FS-288. In control experiments in which activin betaB cDNA was transfected alone or co-transfected with pcDNA vector, no activin B was immunoprecipitated by the anti-HA antibody that recognized FLRG.

In a reverse experiment where activin B was immunoprecipitated by an anti-myc antibody, FLRG, FS-315, and FS-288 all co-immunoprecipitated with activin B. These results further support the specificity of the interaction between FLRG and activin B.

In the cell culture media, secreted FLRG co-immunoprecipitated the secreted, low molecular weight mature form of activin B as demonstrated by the detection of the 14 kDa monomeric betaB protein on the Western blot. In addition, secreted FLRG also co-immunoprecipitated the secreted high molecular weight precursor form of activin B as demonstrated by the detection of the 55 kDa monomeric betaB protein on the Western blot.

Differential expression of FLRG and follistatin.